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APOPTOTIC CELL DEATH IN NEURAL STEM CELLS EXPOSED TO TOXIC STIMULI

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Stockholm 2007

Published by Karolinska Institutet. Printed by Universitetsservice US-AB.

ISBN 978-91-7357-301-6

To my family

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but rather 'Hmm… that's funny'"

- Isaac Asimov (1920 - 1992).

ABSTRACT

Neural stem cells (NSCs) play an important role in the developing nervous system and in the adult brain, where mitotic regions such as the subventricular zone (SVZ) remain active. In spite of the intense ongoing research on NSCs, we still need to expand the knowledge on biochemical regulation by which NSCs undergo cell death in the course of normal physiology or in response to neurotoxic insults. Also, before the full potential of NSCs can be appreciated, it is essential to understand the physiological pathways that control their proliferation and differentiation, as well as the influence of extrinsic factors on these processes. We have studied the general apoptotic machinery in NSCs. As experimental models we used primary cultures of adult NSCs (aNSCs) from the SVZ of the adult rat brain, and the neural stem cell line C17.2, initially derived from the developing mouse cerebellum. Our data show that NSCs undergo apoptosis in response to the pan-kinase inhibitor staurosporine, or to agents inducing oxidative stress such as 2,3-dimethoxy-1,4-naphthoquinone. Exposed cells exhibit apoptotic nuclear morphology, phosphatidylserine translocation to the outer leaf of the plasma membrane, cytochrome *c* release, caspase activation and DNA fragmentation. Additionally our results suggest that extensive oxidative stress causes p53 accumulation and activation of caspase-2, which in turn regulates the mitochondrial apoptotic signaling. Our findings show the importance of the intrinsic mitochondria-mediated pathway in NSC apoptosis induced by toxic stimuli. Both aNSCs and C17.2 cells express the Fas receptor, but exposure to agonistic antibodies fails to induce apoptosis. It is known that Fas not only induces apoptosis, but also can deliver growth stimulatory signals through activation of the extracellular-signal regulated kinase (ERK) pathway. The Fas-induced ERK phosphorylation that we detect in C17.2 cells, suggests that in NSCs Fas may function as a mediator of growth rather than death. There is still little understanding about how neurotoxicants affect the developing nervous system, especially at low-dose exposures. Hence, we have investigated the toxic effects of the environmental neurotoxicants methylmercury (MeHg) and manganese (Mn) in C17.2 cells and primary embryonic cortical NSCs (cNSCs). Our results show that NSCs are more sensitive to both MeHg and Mn than differentiated neuronal or glial cells. Both toxicants induce apoptosis via Bax-activation, cytochrome *c* release, and activation of downstream caspases. In addition, a parallel calpain-dependent cell death pathway could be detected upon MeHg exposure. Remarkably, exposure to MeHg at concentrations lower than observed in cord blood of Swedish pregnant women inhibits spontaneous neuronal differentiation of NSCs, via activation of the Notch signaling pathway. In conclusion this study shows that NSCs are a highly sensitive model system for *in vitro* developmental neurotoxicity studies and offer new perspectives for evaluating the biological significance of low level exposures to neurotoxicants.

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V. **Tamm C, Sabri F, Ceccatelli S.**
Mitochondrial-mediated apoptosis in neural stem cells exposed to manganese.
Submitted to Toxicological Sciences

VI. **Tamm C, Duckworth JK, Hermanson O, Ceccatelli S.**
MeHg inhibits neuronal differentiation of neural stem cells via Notch signalling
Submitted to NeuroReport

ADDITIONAL RELEVANT PUBLICATIONS

I. **Ceccatelli S, Tamm C, Sleeper E, Orrenius S.**
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II. **Johansson C, Tofighi R, Tamm C, Goldoni M, Mutti A, Ceccatelli S.**
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IV. **Ceccatelli S, Tamm C, Zhang Q, Chen M.**
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V. **Vakifahmetoglu H, Olsson M, Tamm C, Heideri N, Orrenius S, Zhivotovsky B.**
DNA damage induced mitotic catastrophe results in apoptosis or necrosis.
Manuscript under revision.

VI. **Akanda N, Tofighi R, Tamm C, Brask J, Elinder F and Ceccatelli S.**
Voltage-dependent anion channels (VDAC) in the plasma membrane play a critical role in apoptosis in differentiated hippocampal neurons but not in neural stem cells.
Manuscript submitted

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LIST OF ABBREVIATIONS

AIF	Apoptosis inducing factor
ADAM	A disintegrin and metalloprotease
PKB	protein kinase B
aNSC	Adult NSC
AP-1	Activator protein 1
Apaf-1	Apoptosis protease activating factor-1
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
BH	Bcl-2 homology
bHLH	Basic helix-loop-helix
Bid	Bcl-2 interacting domain
BIR	Baculoviral IAP repeat
BMP	Bone morphogenic protein
CAD	Caspase-activated DNase
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate-specific protease
CNS	Central nervous system
cNSC	Cortical NSC
CSL	CBF1/Su(H)/LAG1
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DMNQ	2,3-dimethoxy-1,4-naphthoquinone
DMT-1	Divalent metal transporter 1
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERK	Extracellular-regulated kinase
ES	Embryonic stem cells
FADD	Fas-associated death domain-containing protein
FGF	Fibroblast growth factor
FLIP	FLICE (caspase-8) like inhibitor protein
GFAP	Glial fibrillary acidic protein
GSH	Glutathione
H_2O_2	Hydrogen peroxide
Hes	Hairy/Enhancer of Split
HMW	High molecular weight
HSP	Heat shock protein
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
JNK	c-Jun NH_2-terminal protein kinase

LMW	Low molecular weight
MAPK	Mitogen-activated protein kinase
MeHg	Methylmercury
$MnCl_2$	Manganese dichloride
MnTBAP	Mn(III)tetrakis(4-benzoic acid) porphyrin
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGF	Nerve growth factor
NICD	Notch intracellular domain
NSC	Neural stem cell
PARP	Poly(ADP-ribose)-polymerase
PI	Propidium Iodide
PIDD	p53-induced protein with a death domain
PS	Phosphatidylserine
RAIDD	RIP associated ICH/CED3 homologous protein with death domain
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
SGZ	Subgranular zone
siRNA	Short interfering RNA
Smac	Second mitochondria-derived activator of caspases
STS	Staurosporine
SVZ	Subventricular zone
TACE	Tumor necrosis factor alpha converting enzyme
tBid	Truncated Bid
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand receptor
TUNEL	TdT-mediated dUTP-biotin nick end labeling
XIAP	X chromosome-linked inhibitor of apoptosis
ZIP8	Zrt-like, Irt-like protein 8

INTRODUCTION

NEURAL STEM CELLS

Cells can be defined as "stem cells" when they fulfill two key criteria;

- Self-renewal – The cells have to be able to divide and proliferate in a way that gives rise to at least one new stem cell. This can be achieved by either symmetric or asymmetric cell division. Symmetric cell division results in two daughter cells exhibiting similar properties as the dividing cell. This kind of cell division exponentially expands the cell population. Asymmetric cell division, on the other hand, generates two different daughter cells. Most often one cell remains a *de facto* stem cell, while the other becomes a progenitor cell with more lineage restrictions. During neurodevelopment it is thought that at early stage there is mainly symmetric cell division (Caviness et al., 1995), while later on it shifts to asymmetric cell division with the generation of cells differentiating into post-mitotic neural cells and new stem cells.

- Multi-lineage differentiation – Many cells can proliferate and bring about descendants of the same cell type (e.g. fibroblasts and myoblasts). Stem cells are able to differentiate into two or more cells characteristic of the tissue from which they have been isolated. For example, a neural stem cell (NSC) can differentiate into cells present within the central nervous system (CNS); such as neurons, astrocytes and oligodendrocytes (Johe et al., 1996; Reynolds and Weiss, 1992).

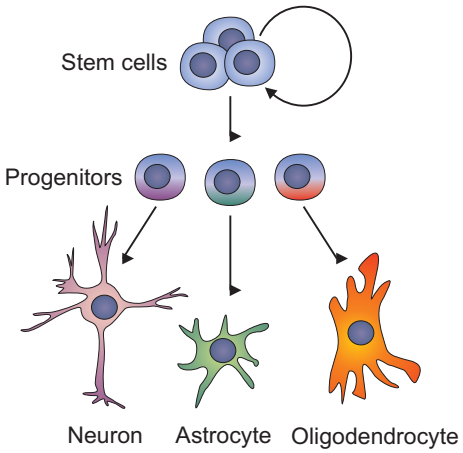

Figure 1. Neural stem cells proliferate and divide symmetrically, thus increasing the stem cell population. When dividing asymmetrically or differentiating, the stem cells form developmentally restricted precursor cells, which finally differentiate into either mature neurons, astrocytes or oligodendrocytes.

This minimal definition has lead to a classification of cells, which possess these properties based on the tissue or organ from where the cells have been isolated. Hence, NSCs are derived from the CNS, whilst embryonic stem cells are derived from the blastocysts. Tissue specific stem cells, other than neural stem cell, have been identified in the hematopoietic system, pancreatic islets, liver, intestine and skin. Stem cells have been described in a wide range of tissues, and these cells have been

shown to be able to divide, self-renew and differentiate into tissue-characteristic cells (Cai and Rao, 2002). In their respective organs, the stem cells reside in designated stem cell niches, which provide a controlled environment for proliferation and differentiation. The NSC niches include characteristic cytoarchitectures of the specific anatomical areas of the CNS, and several other cellular and molecular prerequisites, such as proximity to the cerebrospinal fluid and blood vessels, cell to cell interactions and signaling, and unique basal lamina and extracellular matrix (Doetsch, 2003). It is generally agreed that NSCs exist in a variety of developmental stages. So far multipotent fetal NSCs have been found and isolated from numerous different brain regions of the embryonic CNS, such as the olfactory bulb, subventricular zone, hippocampus, cerebellum, cerebral cortex and spinal cord (Davis and Temple, 1994; Lee et al., 2005; Marmur et al., 1998; Pagano et al., 2000; Palmer et al., 1997; Reynolds et al., 1992; Uchida et al., 2000). Although some suggest that not all CNS stem cells can be identified by the expression of a single protein (Dahlstrand et al., 1995; Kukekov et al., 1997), the class VI intermediate filament protein nestin (an acronym for neuroepithelial stem cell protein) (Lendahl et al., 1990), has been shown to be transiently expressed in most neural stem or progenitor cells. Upon differentiation nestin is downregulated and replaced by other intermediate filament proteins such as glial fibrillary acidic protein (GFAP) in astrocytes or neurofilaments in neuronal cells (Dahlstrand et al., 1995; Lendahl et al., 1990; Messam et al., 2000).

During early brain development NSCs take on positional identity within the neural tube via adjacent tissue-secreted morphogenic signaling. Positional characteristics along the anteroposterior axis are specified by fibroblast growth factors (FGF), Wnt and retinoid family ligands, whereas along the dorsoventral axis they are specified by the antagonistic actions of bone morphogenic proteins (BMPs), transforming growth factor β and Sonic Hedgehog (Altmann and Brivanlou, 2001). Some of these morphogens are also mitogens that promote proliferation, proposedly via the induction of positional identity genes. For example it has been shown that the expression of transcription factors Pax6, Emx2, Lhx2, and Foxg1 is required for the proliferation of cortical precursors (Estivill-Torrus et al., 2002; Heins et al., 2001; Monuki et al., 2001). Moreover, FGF2 promotes proliferation of cortical NSCs *in vivo* (Vaccarino et al, 1999), but is also, together with epidermal growth factor (EGF), the only ligand known to promote NSC proliferation *in vitro* (Ford-Perriss et al., 2001). Part of the NSC proliferation signaling involves the suppression of programmed cell death. For example, the absence of retinoic acid, erythropoietin or Notch receptor signaling markedly decreases proliferation but also increases apoptosis during early gestation (Lutolf et al., 2002; Schneider et al., 2001; Shingo et al., 2001). Notch signaling is also known to inhibit neuronal differentiation and maintain NSCs in a proliferative state (Gaiano and Fishell, 2002). Activation of Notch leads to upregulation of Hes1 and Hes5, transcription factors with a conserved basic helix-loop-helix (bHLH) domain in their DNA binding region. Although Notch

and Notch ligands are not expressed during early CNS development, NSCs obtained from later developmental stages seem to depend on Notch signaling to stay alive, proliferative and undifferentiated (Hitoshi et al., 2004). Hes1 and Hes5 upregulation induces NSC proliferation and repression of the neurogenic bHLH genes (Nakamura et al., 2000; Ohtsuka et al., 2001). The neurogenic bHLH transcription factors, such as Math1/2, Ngn1/2, Mash1 and NeuroD, are required for promoting neurogenesis and inhibition of gliogenesis (Farah et al., 2000; Nieto et al., 2001). These neurogenic bHLH genes also seem to be essential for sustaining the stemness in nearby NSCs via Notch signaling (Kageyama et al., 2005). Overall, during development NSCs change their competency and sequentially give rise to different cell types. Thus, the maintenance of NSCs until the late developmental stages is essential to warrant the correct magnitude and diversity of cells. NSCs *in vitro* can be induced to differentiate simply by removing mitogens, which will lead to spontaneous differentiation to various proportions of neurons, astrocytes and oligodendrocytes (Johe et al., 1996). By adding various growth factors, such as the platelet derived growth factor, cilliary neurotrophic factor, BMPs, or thyroid hormone T3, the commitment of the differentiating cells can be altered and directed depending on the developmental stage of the NSCs (Gross et al., 1996; Johe et al., 1996; Li et al., 1998b; Panchision and McKay, 2002).

ADULT NEURAL STEM CELLS

In contrast to formerly held beliefs that the adult brain is a static system without scope for cell replacement and regeneration, there is now good evidence for the differentiation of neural cells from stem cells in the adult brain with the ability to integrate into the complex circuitry of the CNS (see Gross, 2000). Scientists started to suspect active mitosis in the adult brain in the years spanning from beginning of the 20th century to the 1960's (Bryans, 1959; Hamilliton, 1901). Due to methodological limitations it was not confirmed until the middle of the 1960's when Altman started to label dividing cells with [^{3}H]-thymidine incorporated into the newly formed DNA (Altman, 1962; Altman and Das, 1965). Kaplan combined this labelling with electron microscopy (Kaplan and Hinds, 1977) and showed that neurogenesis occurred in the adult brain of rodents. In the adult brain neurogenesis has been characterized in at least two areas: the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Taupin et al., 2000), and the olfactory bulb (Hinds, 1968; Lois and Alvarez-Buylla, 1994). The source of these newly formed neuronal cells have also been subject of investigation and the two brain regions, which primarily has been ascribed the formation of the neurons, are the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Gage et al., 1998) and the subventricular zone (SVZ) of the lateral ventricle (Reynolds and Weiss, 1992). The SVZ is the larger germinal zone and is situated adjacent to the ependyma of the lateral ventricle wall. Cells from this area have been found to migrate to the olfactory bulb where they differentiate into mature granule cells and periglomerular cells, the two foremost interneurons in this part of the brain (Alvarez-Buylla and Garcia-Verdugo, 2002). Since it has been estimated

that approximately 30 000 new interneurons are formed everyday in the adult mouse brain all through life, this level of neurogenesis led to the belief that there exists asymmetrically dividing self-renewing stem cells within the SVZ (Lois and Alvarez-Buylla, 1994).

When cultured, cells from the SVZ grow adherently in cell culture dishes in serum-free medium supplemented with EGF and/or FGF. These act as mitogens for the cells both *in vitro* (Gritti et al., 2002; Reynolds et al., 1992) and *in vivo* (Craig et al., 1996; Li and DiCicco-Bloom, 2004). After awhile the cells detach from the substrate and produce characteristic free floating aggregates of closely grouped cells, referred to as neurospheres. In their proliferative state, cells in the neurosphere express the stem cell-specific intermediate filament protein nestin. Immunohistochemical staining of brain sections also show nestin-expressing cells in the SVZ, as well as the ependymal layer of the lateral ventricle (Doetsch et al., 1997; Morshead et al., 1994). Upon removal of EGF and FGF, cells can differentiate into neurons, astrocytes and oligodendrocytes (Gage et al., 1995; Reynolds et al., 1992). Nestin-expression, self-renewal and multi-lineage differentiation properties confirm that these cells are tissue specific stem cells, although clonal analyses of neurosphere cells have shown that based on these criteria, only ~16% of the cells can be considered to be *de facto* stem cells (Gritti et al., 1996). The majority of the remaining cells are stem cell-derived progenitors with a more limited proliferation and differentiation potential (Mayer-Proschel et al., 1997). However, it still unclear which cells in the SVZ are the resident adult NSCs (aNSCs). Several cell types has been proposed, including astrocytes (Doetsch et al., 1999), multiciliated ependymal cells (Johansson et al., 1999) and subependymal cells (Morshead et al., 1994).

The wide interest in NSCs is mainly based on the perceived therapeutic potential these cells could offer in repair after brain injury or in the treatment of neurodegenerative diseases. Brain injuries, e.g. subsequent to stroke, have been shown to stimulate proliferation in both the SGZ and the SVZ with an ensuing migration to the damaged area (Arvidsson et al., 2002; Kokaia and Lindvall, 2003; Parent, 2003). Alterations in the aNSC-containing brain areas have also been seen in chronic degenerative neurological disorders, such as Huntington's disease and Alzheimer's disease. In these diseases proliferation and neurogenesis are increased (Curtis et al., 2003; Jin et al., 2004). On the other hand, in Parkinson's disease proliferation in these areas has been shown to be impaired (Hoglinger et al., 2004). All considered, adult neurogenesis might be a fundamental compensatory response for self-repair in the adult CNS. The possibility of harvesting stem cells that can be amplified in culture and later used for repair and regeneration in cell replacement therapies, has been the driving force that has made stem cells currently one of the hottest topics in science.

CELL DEATH

Normal development and maintenance of cell homeostasis, as well as numerous injuries and various diseases, are associated with cell death. Until recently most of the attention in the cell death field has been focused on the investigation of one pathway by which cells die, namely, apoptosis (from the Greek, 'falling off'). The term was first coined by Kerr, Wyllie and Currie (Kerr et al., 1972) but the morphology of apoptosis was described earlier by several investigators (reviewed by Lockshin and Zakeri, 2001; Vaux, 2002), and has been almost synonymous for cell death. Apoptosis is an active and energy-dependent process that occurs at single cell level. This mode of regulated cell death is vital for example in embryogenesis, general tissue homeostasis and for the development and function of the immune system (Thompson, 1995). Inadequate or excessive apoptotic cell death is associated with several diseases. Cancer, rheumatoid arthritis and lymphoproliferative diseases are pathological situations with decreased apoptosis, while in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease increased apoptosis has been reported (see Fadeel et al., 1999; Kerr et al., 1994; Thompson, 1995). Morphological and biochemical hallmarks of apoptosis have been well characterized (Hengartner, 2000; Kerr et al., 1972). Cells typically round up, form plasma membrane blebs, undergo zeiosis (a boiling-like appearance due to rapid bleb formation) and chromatin condensation, and bud off condensed membrane-packaged vesicles called apoptotic bodies. DNA is cleaved between the chromatin subdomains generating high molecular weight (HMW) fragments of 300 and 50 kbp, and at internucleosomal sites generating low molecular weight (LMW) fragments of 180 bp (Oberhammer et al., 1993; Tomei et al., 1993). Phosphatidylserine, normally facing the inside of the cell, flips to the outer leaf of the plasma membrane where it is recognized by neighbouring cells and macrophages, which engulf the apoptotic bodies before extracellular leakage can occur and thus prevent an inflammatory response (see Henson et al., 2001). The activation of a family of cysteine proteases, i.e. caspases, and DNases occurs mainly through two main pathways. These are the extrinsic pathway, induced by the activation of cell-surface death receptors such as CD95 (Fas/Apo-1), or the intrinsic pathway mediated by the mitochondrial release of pro-apoptotic proteins such as cytochrome *c* (see below). There have also been reports suggesting a third pathway causing caspase activation, originating from the endoplasmic reticulum (ER) (Morishima et al., 2002; Rao et al., 2002). Other organelles, e.g. the nucleus and the Golgi apparatus, are also linked to the apoptotic machinery via damage sensors (Green and Kroemer, 2005).

In addition to apoptosis, several other cell death pathways have been described. According to a recent classification, eight different modes of cell death have been delineated, but there are researchers proposing up to 11 different pathways in mammals (Kroemer et al., 2005; Melino et al., 2005). A few of these will be described here. In contrast to apoptosis, it was until recently believed that necrosis (from the Greek, 'dead body') was a passive and energy-independent form of cell

death often caused by serious injury or other severe circumstances, which compromised the integrity of the cell membrane. As a result of unrestrained water and ion influx, necrotic cells burst and the cell content is released into the extracellular space. Enzymes, such as proteases, lipases and nucleases, as well as by-products from cell metabolism, induce further injury to the surrounding cells and tissue with subsequent inflammation (Majno and Joris, 1995). Recently, it has been suggested that necrosis not only occurs during pathological events, but is also involved in some physiological processes. For example, during renewal of the small intestine, both apoptosis and necrosis of enterocytes contribute to the cell loss (Oppenheim et al., 2001). In addition, growing evidence suggests that necrotic cell death can be programmed and regulated (Vande Velde et al., 2000). Although the precise mechanism of programmed necrosis has yet to be elucidated, several signaling processes have been identified to initiate necrosis, e.g. RIP kinase, Ca^{2+}, ceramide, JNK/p38, and excessive activation of PARP (Mills et al., 2004; Okada and Mak, 2004; Proskuryakov et al., 2002). Programmed cell necrosis has been associated with the pathophysiology of a number of diseases, such as vascular-occlusive disease, neurodegenerative disorders, infections, inflammatory diseases, and cancer, as well as toxicant exposure. The distinction between apoptosis and necrosis is not always clear and there are many cases where the same insult induces both modes of cell death, independently or in parallel. This depends on the duration and severity of the insult, and cell sensitivity (Ankarcrona et al., 1995).

Autophagy (from the Greek, 'self eating'), is often referred to as "type II programmed cell death" where "type I" refers to apoptosis, and has been described during development in several organisms (Clarke, 1990; Schweichel and Merker, 1973). Under normal physiological conditions, autophagy is involved in the turnover of long-lived proteins and organelles and has been shown to occur at basal levels in most tissues. This evolutionary conserved lysosomal pathway promotes cell adaptation and survival during stress, such as starvation (see Kroemer and Jaattela, 2005). The most prominent morphological feature is the appearance of double- or multiple-membrane enclosed vesicles, so called autophagosomes, in the cytoplasm, which engulf portions of the cytoplasm and/or organelles such as the mitochondria and the ER. These vesicles then fuse with lysosomes, delivering their content for degradation and recycling by catabolic enzymes (Ohsumi, 2001; Reggiori and Klionsky, 2002). However, excessive autophagy leads to cell death. In principle, autophagic activity above a certain threshold leads to an irreversible type of cellular atrophy, causing a total collapse of cellular functions (Lum et al., 2005). Cellular hallmarks of apoptosis and autophagy frequently occur together or in close temporal proximity, and inhibition of apoptosis has been shown to induce autophagic cell death (Chautan et al., 1999; Yaginuma et al., 2001). At the molecular level, signaling pathways including Beclin-1, phosphatidylinositol 3-kinase, the kinase target of rapamysin, and death-associated protein kinase, have been shown to be involved in autophagy initiation (Kelekar, 2005). However, it is still debated whether autophagy plays a role

in cell death or is just keeping the cell alive under stress conditions before their demise.

Originally, mitotic cell death, often referred to as mitotic catastrophe, was described as the main mode of cell death induced by ionizing radiation (Jonathan et al., 1999). However, it has also been shown to be initiated by exposure to microtubule stabilizing or destabilizing agents, various anticancer drugs and deficient cell cycle checkpoints. This mode of cell death is thought to occur during, or shortly after, dysregulated or failed mitosis, and to be fundamentally different from apoptosis (Roninson et al., 2001). Morphologically, mitotic catastrophe differs from apoptosis in that it is characterized by the formation of nuclear envelopes around individual clusters of miss-segregated, uncondensed chromosomes, thus giving rise to multinucleated giant cells. Although some reports suggest that mitotic catastrophe shares several biochemical hallmarks of apoptosis, in particular mitochondrial membrane permeabilization and caspase activation (Castedo et al., 2004), Bcl-2 overexpression and caspase inhibition do not prevent mitotic catastrophe and the accumulation of giant multinucleated cells (Lock and Stribinskiene, 1996; Roninson et al., 2001). Conversely, recently we suggested that, despite its distinctive morphology, mitotic catastrophe may represent a pre-stage of other cell death modes, such as apoptosis and/or necrosis (Vakifahmetoglu et al., 2007), determined by the molecular profile of the cells (Chu et al., 2004; Nitta et al., 2004).

THE EXTRINSIC AND INTRINSIC APOPTOTIC PATHWAYS

The two most investigated pathways of apoptosis in mammalian cells are the death receptor-mediated (extrinsic) pathway, involving activation of so called death receptors on the plasma membrane, and the intrinsic pathway, which involves mitochondria-mediated signaling (figure 2).

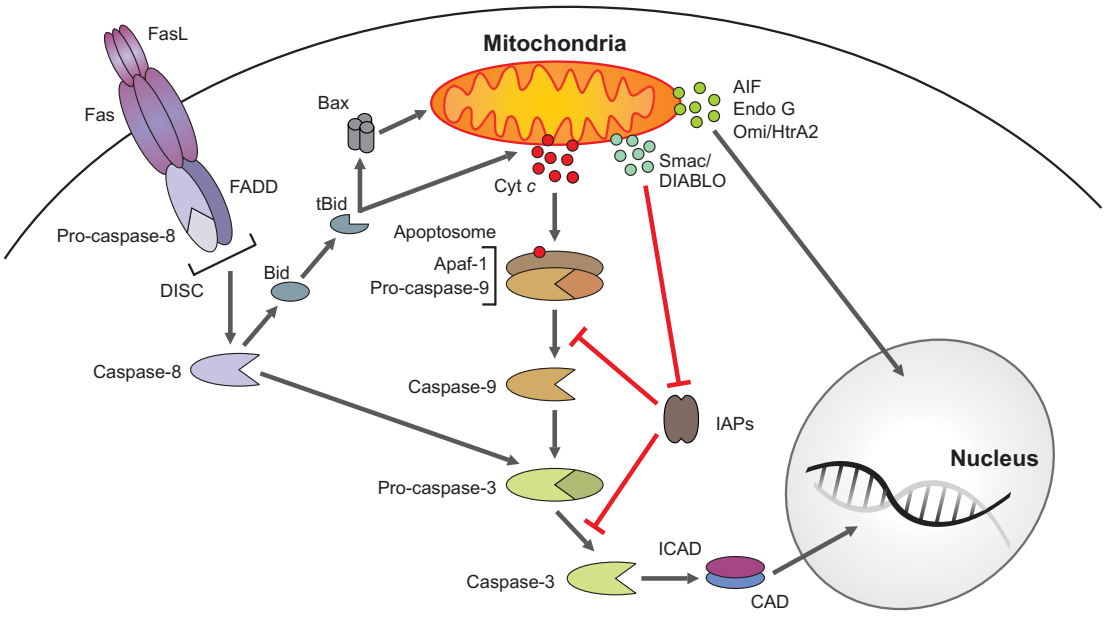

Figure 2. An overview of the extrinsic and intrinsic cell death pathways.

The death receptor-activated extrinsic pathway

Death receptors are expressed on the cell surface of many different cell types and can initiate apoptotic cell death when activated by their cognate ligands (Itoh et al., 1991; Oehm et al., 1992). The receptors, including CD95 (Fas/Apo-1), TNFR1 and TRAIL, belong to the tumor necrosis factor (TNF) superfamily of plasma membrane death receptors (Sartorius et al., 2001). Upon ligand binding they aggregate into trimers and recruit adaptor proteins, such as Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) containing proteins, via interactions through the death domains of the receptors (see Schmitz et al., 2000). Further, FADD or TRADD proteins recruit initiator procaspase-8 or -10 (see below) via their death effector domains (DED). This assembly of proteins is called the death-inducing signaling complex (DISC) in which the procaspases are autocatalytically processed to active caspases (Medema et al., 1997). The following events are cell type specific. In so called "type I" cells, the apical caspases initiate a caspase cascade with succeeding activation of effector caspases-3 and -7. In "type II" cells active caspase-8 cleaves Bid, a pro-apoptotic Bcl-2-family member (see below). Truncated Bid (tBid) translocates to the mitochondria and initiates the intrinsic apoptotic pathway (Li et al., 1998a; Luo et al., 1998).

The mitochondrial-mediated intrinsic pathway

Apart from being the "power plant" of the cell, the mitochondria are also one of the main organelles for apoptosis. Mitochondrial membrane permeabilization, and the subsequent release of pro-apoptotic molecules, may initiate apoptosis by numerous mechanisms. This process is tightly regulated by Bcl-2-family proteins. Although several proteins release from mitochondria, e.g. apoptosis inducing factor (AIF), Second mitochondrial activator of caspases (Smac)/direct IAP binding protein with low pI (DIABLO), Endonuclease G and Omi/HtrA2 (van Gurp et al., 2003), the most well-characterized molecule is cytochrome *c*, a protein that upon release initiates the intrinsic apoptotic pathway. Cytochrome *c* resides in the mitochondrial inter-membrane space and normally functions as an electron transporter between complexes III and IV in the mitochondrial respiratory chain. When released into the cytosol, cytochrome *c* associates with apoptotic protease-activating factor-1 (Apaf-1) via a caspase recruitment domain (CARD). Hydrolysis of dATP/ATP enables oligomerization of Apaf-1 and simultaneously the binding of procaspase-9 to the exposed CARD, thus forming a complex referred to as the apoptosome (see Budihardjo et al., 1999). Autocatalytic activation of procaspase-9 within the apoptosome initiates the cleavage and activation of caspases-3 and -7. One notable caspase-3 substrate is ICAD (inhibitor of caspase-activated DNAse). The caspase-dependent ICAD cleavage releases active CAD (caspase-activated DNAse), which translocates into the nucleus and induces DNA fragmentation. The flavoprotein AIF hold a strong homology to several oxidoreductases (Daugas et al., 2000; Lorenzo et al., 1999). Under normal conditions it is restricted to the mitochondria (Susin et al., 1999), where it is presumed to be a vital protein in the respiratory chain. In certain

experimental models, apoptotic triggering induces AIF release and its translocation from the mitochondria to the nucleus, where it involves via unknown mechanisms in chromatin condensation and generation of HMW DNA fragments (Susin et al., 1999; Vieira and Kroemer, 1999; Yu et al., 2002).

CASPASES

One of the main features of apoptosis is the proteolytic cleavage of several key proteins important for maintaining cytoskeletal cell structure, DNA-repair system and cell cycle regulation. This is primarily achieved by a family of evolutionary conserved cysteine proteases called caspases (for cysteinyl aspartate proteases) (Alnemri et al., 1996; Bratton et al., 2000; Samali et al., 1999). Over the years a number of caspases have been identified in various mammalian and non-mammalian species. Eleven caspases have been described in human, ten in mouse, four in chicken, four in zebrafish, seven in *Drosophila melanogaster* and four in *Caenorhabditis elegans* (Lamkanfi et al., 2002). Many of the known caspases have been found to have dual functionality in both apoptotic and non-apoptotic signaling. The activation of caspases and their cleavage of substrates in the absence of cell death have more and more become a hot research field. Reports of substrates such as cytokines, kinases, transcription factors and polymerases, support additional functions for caspases in the regulation of cell survival, proliferation, differentiation and inflammation (see Lamkanfi et al., 2007). The therapeutic inhibition of caspases to prevent cell death could, therefore, have wider repercussions than initially perceived. Under normal conditions caspases are inactive pro-enzymes (zymogens), and although they demonstrate a low activity, procaspases are restrained by various regulatory molecules. Upon pro-apoptotic stimuli procaspases can be processed and activated (Earnshaw et al., 1999). Zymogen cleavage is not an obligatory requirement for activation, but cleaved fragments of all activated caspases can be found in apoptotic cells (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). All pro-caspases contain four domains: an N-terminal pro-domain of variable length; a large subunit (17-21 kD); a small subunit (10-13 kD); and a linker region between the subunits. During activation the pro-domain and the linker region are proteolytically cleaved at specific aspartate residues, leaving a heterodimer of the small and large subunits. The active caspases consists of a tetramer composed of two of these heterodimers (Liang and Fesik, 1997). Caspases, apart from caspase-2 (see below), recognize specific tetrapeptide motifs containing aspartatic acid of which they cleave the C-terminal peptide bond (Nagata, 1997).

Caspases can be divided into two major groups based on the length of the pro-domains. Caspases with a relatively long pro-domain (procaspases-2, -8, -9 and -10), which contains either DEDs or CARDs, are recruited and autocatalytically activated in a still to be defined “proximity induced” activation mechanism in large multimeric complexes. So far four caspase complexes have been identified; the apoptosome (Cain et al., 2002; Hengartner, 1997), the DISC complex (Peter and Krammer, 2003),

the PIDDosome (Tinel and Tschopp, 2004), and the caspase-1-containing inflammasome (Martinon and Tschopp, 2004). These caspases, which supply a link between cell signaling and the apoptotic machinery, are called initiator (or apical) caspases. The procaspases with short pro-domains (caspases-3, -6 and -7) are deficient in recruitment domains and thus unable to self-activate. Instead, they are further processed by the apical caspases. These caspases are referred to as effector caspases, due to their direct downstream action on structural and regulatory proteins (Thornberry and Lazebnik, 1998). This cascade-like activation of caspases is thought to play a critical role in the cell death process of apoptosis.

Activation of caspase-8 is an essential factor in the extrinsic apoptotic pathway, which happens upon ligand binding to death receptor (see above). Caspase-8 can subsequently activate caspase-3 directly or via cleavage of Bid with the subsequent activation of the intrinsic mitochondrial pathway. The main caspase component of the intrinsic mitochondrial pathway is caspase-9. As mentioned above, the release of apoptotic factors, such as cytochrome *c,* promotes formation of a septameric apoptosome that recruits and activates procaspase-9. Apoptosome-associated active caspase-9 will subsequently cleave and activate procaspase-3. The effector caspases, such as caspase-3, can also be cleaved and activated by other proteases, such as cathepsins, calpains and granzymes (Johnson, 2000). Among the effector caspases, caspase-3 cleaves the majority of the cellular substrates in apoptotic cells (Porter and Janicke, 1999), although the substrate specificity is highly shared with caspase-7 (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). By initiating degradation of structural and regulatory proteins, effector caspases cause membrane blebbing, chromatin condensation and fragmentation of DNA, which all are hallmarks of apoptosis. Effector caspases have also been suggested to take part in amplifying mitochondrial caspase-activation signaling (Lakhani et al., 2006).

Caspase-2 was one of the first cloned caspases, but its physiological function still remains a matter of considerable debate. Its pro-form is the only inactive caspase found in the nuclei (Shikama et al., 2001; Zhivotovsky et al., 1999), and subcellular fractionation studies have demonstrated its presence also in the Golgi complex, mitochondria, and cytosol (O'Reilly et al., 2002; Ren et al., 2005). Caspase-2 shares sequence homology with the initiator caspases, however the cleavage specificity of caspase-2 is more closely related to the effector caspases. Procaspase-2 contains a CARD, through which it is recruited to a high molecular weight complex, the so called PIDDosome, containing RAIDD (RIP associated ICH/CED3 homologous protein with death domain) and PIDD (p53-induced protein with a death domain) (Lin et al., 2000; Tinel and Tschopp, 2004). In contrast to other known caspases that have tetrapeptide cleavage specificity, caspase-2 requires a pentapeptide motif (VDVAD). Therefore, of the close to 400 proteins presently known to be processed by caspases, only a few can be cleaved by caspase-2. So far, in addition to itself, active caspase-2 has been shown to be able to process the Golgi complex-specific protein golgin-160

(Mancini et al., 2000), αII-spectrin (Rotter et al., 2004), and PKCδ (Panaretakis et al., 2005). As a response to DNA-damage, caspase-2 has been demonstrated to cause mitochondrial permeabilization and release of proapoptotic factors, such as cytochrome *c* (see Troy and Shelanski, 2003). Inhibition of caspase-2 has been shown to prevent cell death after exposure to various stimuli, including chemotherapeutic drugs (Lassus et al., 2002; Robertson et al., 2002).

CALPAINS

Calpains are a family of cytoplasmic neutral cysteine proteases, which have been found to be ubiquitously and tissue-specifically expressed. Currently six different calpains have been described and they are divided into two groups: μ-calpains and m-calpains. In many models of apoptosis intracellular Ca^{2+}-level is increased. This can lead to the activation of calpains, which require Ca^{2+} for their optimal activity (Kass and Orrenius, 1999; Saido et al., 1994). When activated, calpains are translocated to phospholipid membranes where they undergo autolysis, which lowers their intrinsic Ca^{2+}-requirements (Chan and Mattson, 1999). The two groups of calpains have the same substrate specificity, but they can easily be distinguished on the basis of their Ca^{2+} requirement. μ-calpains are activated at micromolar concentrations, whereas m-calpains requires millimolar concentrations (Johnson, 2000). A large variety of proteins have been identified as calpain substrates, including procaspase-3, -9 and the cytoskeletal protein fodrin (Hirai et al., 1991; Vanags et al., 1996). Most of the substrate proteins are cytoskeletal proteins or proteins associated with cell membranes. Calpains have therefore been hypothesized to play an important role in the destruction of cellular architecture during apoptosis, but also during normal proliferation that requires rearrangement of the cytoskeleton (Johnson, 2000). In healthy cells calpains are bound by the calpain-specific inhibitor protein calpastatin, which in turn is thought to be cleaved during apoptosis by activated caspases (Porn-Ares et al., 1998; Wang et al., 1998). Lately calpains have been shown to be able to cleave Bid, which leads to activation of the mitochondrial intrinsic pathway (Chen et al., 2001) as described above.

REGULATION OF APOPTOSIS

There are several protein families engaged in the control of apoptosis, such as inhibitor of apoptosis proteins (IAPs), heat-shock proteins (HSPs) and the Bcl-2 family (e.g. Deveraux and Reed, 1999; Gross, 2001; Xanthoudakis and Nicholson, 2000). To date, eight human IAPs have been identified: including XIAP, c-IAP1, c-IAP2 and Survivin (see Callus and Vaux, 2007). Among the currently known IAPs are only XIAP (X chromosome-linked inhibitor of apoptosis), c-IAP-1 and -2 physically able to interact with caspases and inhibit their activity (Vaux and Silke, 2005). However, only XIAP inhibits caspases at physiological concentrations. Structural studies have shown that the N-terminal Baculoviral IAP repeat (BIR) 2 linker region of XIAP binds to the catalytic site of active caspase-3 and -7, while regions close to the BIR3 region act on caspase-9 (Chai et al., 2001; Takahashi et al.,

1998). XIAP and cIAP-1 have been shown to be processed and inactivated by caspases to ensure the induction of apoptosis (Deveraux and Reed, 1999). In addition, Smac/DIABLO and HtrA2, released from the mitochondria during apoptosis, can bind to the BIR domains of XIAP and prevent the inhibition of caspase activity (Vaux and Silke, 2003). HSPs have both pro- and anti-apoptotic properties. For example, Hsp60 and Hsp10 have been shown to bind to procaspase-3 and promote its activation, while Hsp70 inhibits the activation of caspase-9 downstream of the mitochondria-mediated pathway (Beere et al., 2000; Bruey et al., 2000; Samali et al., 1999). Additionally, Hsp27 prevents the formation of the apoptosome complex by binding to cytosolic cytochrome *c*, subsequently inhibiting its interaction with Apaf-1 (Bruey et al., 2000).

The Bcl-2 protein family is the major and best described group of apoptosis-regulating proteins. They have been shown to regulate the release of proteins by affecting the permeability of the outer mitochondrial membrane and the endoplasmic reticulum (Sharpe et al., 2004). The Bcl-2 family can be recognized by the presence of conserved sequence motifs, known as Bcl-2 homology (BH) domains 1 to 4, and is divided into two groups: anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-w, A-1/Bfl-1 and Mcl-1) and pro-apoptotic (Bax, Bak, Bcl-X_S, Bad, Bid, Puma and Noxa) (e.g. Gross et al., 1999; Tsujimoto, 1998). The anti-apoptotic Bcl-2 family members can inhibit cell death by sequestering and neutralizing the pro-apoptotic Bcl-2 family members. The BH1, BH2 and BH3 domains in BCL-X_L are in close proximity and form a hydrophobic pocket that can hold a BH3 domain of the pro-apoptotic members. By sequestering BH3-only proteins, such as Bid, Bad, and Bim, which are a subgroup of pro-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L are believed to maintain the outer mitochondrial membrane integrity and consequently prevent the activation of the Bax and Bak (Sharpe et al., 2004). Under normal conditions Bax exists as monomer in the cytosol or loosely bound to the mitochondria. During early stages of apoptosis Bax oligomerizes (Antonsson et al., 2001; Tan et al., 1999), translocates to the mitochondria (Hsu et al., 1997; Saikumar et al., 1998), and inserts into the outer mitochondrial membrane (Goping et al., 1998). The mitochondrial membrane protein Bak resides as monomers in healthy cells and has been shown to oligomerize and co-localize with Bax during apoptosis. Under healthy conditions pro- and anti-apoptotic Bcl-2 family proteins heterodimerize and antagonize one another's function (Oltvai et al., 1993). Shifts in the ratio between pro- and anti-apoptotic proteins that will favor apoptosis, results in the release of cytochrome *c* and AIF. A shift in the other direction, with an increased expression of Bcl-2 and/or Bcl-X_L, has been shown in several cancers. Bid has been shown to be cleaved by numerous proteases, such as caspase-8 (Li et al., 1998a; Luo et al., 1998), granzyme B (Barry et al., 2000), lysosomal enzymes (Stoka et al., 2001), and calpains (Chen et al., 2001), in response to a variety of apoptotic stimuli. Cleaved Bid or tBid, translocates to the mitochondria where it exerts many different functions. It has been shown to tie up anti-apoptotic Bcl-2 family members, as well as directly associate

with Bax, cause mitochondrial permeabilization and initiate Bax or Bak oligomerization (Eskes et al., 2000; Kuwana et al., 2002; Wei et al., 2000).

In addition to the apoptotic inhibitors described above, most cells also express inhibitors of caspase-8/FLICE activation called cFLIPs (cellular FLICE inhibitory proteins) (Irmler et al., 1997). cFLIP is a caspase-8-like protein that lacks both the catalytic site and the substrate binding pocket. cFLIP is upregulated via NFκB signaling and promotes cell survival via DED-DED interactions with FADD, thus inhibiting the recruitment and activation of procaspase-8 at the DISC complex. Recent data suggest that death receptor-mediated JNK signaling activates the Itch ubiquitin ligase, which ubiquitinates cFLIP for proteosomal degradation and thus sensitizes the cell to extrinsic ligand mediated cell death (Chang et al., 2006).

NEURAL STEM CELLS AND CELL DEATH

NSCs die by apoptosis in considerable numbers both during development (Acklin and van der Kooy, 1993; Rakic and Zecevic, 2000; Slack et al., 1995; Thomaidou et al., 1997) and in adulthood as a result of regular cell turnover (Levison et al., 2000). One of the most generally accepted mechanisms regarding developmental apoptosis is the competition among cells for limited supply of neurotrophic factors (see Burek and Oppenheim, 1996). Analyses of embryonic mice with targeted gene disruption of Bcl-X_L, Bax, Apaf-1, caspase-3 and caspase-9 have shown that a fully functional intrinsic apoptotic pathway is needed in neural stem/progenitor cells during development (Cecconi et al., 1998; Deckwerth et al., 1996; D'Sa-Eipper and Roth, 2000; Kuida et al., 1996; Motoyama et al., 1995; Roth et al., 1996; Yoshida et al., 1998). In the adult, studies have shown that Bax and Bak influence the number of NSCs in the mouse brain (Lindsten et al., 2003). The presence of death receptors, such as CD95 (Fas/Apo-1) and its transmembrane ligand, has been described in both the developing central and peripheral nervous system (Zou et al., 2000). The Fas-FasL system has been shown to be active in the developing rat cerebral cortex during the peak of apoptosis (Cheema et al., 1999). It has also been shown that FasL triggers programmed cell death in embryonic motorneurons (Raoul et al., 1999). So far little is known about the mechanism of cell death in neural stem cells exposed to different kinds of toxic insults.

NEUROTOXICITY

Generally neurotoxicity defines structural and/or functional alterations of the nervous system, induced by endogenous or exogenous factors such as chemical, biological, or physical agents (Philbert et al., 2000; Tilson et al., 1995). The complexity and the special features of the nervous system make it particularly vulnerable to insults of various origins. To maintain the membrane polarization and repolarization conductance required for normal neuronal function, a high demand of energy is needed. To meet this demand, the nervous system has a very high metabolic rate that is dependent on continuous aerobic glycolysis. As a consequence, the nervous system

is very sensitive to any aberrations in the supply of glucose and oxygen, of which the latter accounts for almost 20% of the total amount consumed by the body (see Heiss, 1981). The high oxygen consumption, together with a moderate level of antioxidant activity and a high quantity of polyunsaturated fatty acids, render the nervous system particulary vulnerable to oxidative stress (see Evans, 1993).

The adult brain is protected by the blood-brain barrier, an anatomical structure formed by specialized endothelial cells with tight junctions. Consequently, in the adult, although some toxicants can cross the barrier by passing through the membranes of the endothelial cell by diffusion or by active transport, many toxic agents are prevented to enter the brain. In the developing nervous system the blood-brain-barrier is not fully developed until roughly 6 month of age in humans (Risau and Wolburg, 1990), which predisposes fetuses and young infants to brain injuries by insults that do not affect the adult nervous system.

Brain development occurs in different phases and each developmental stage is reached according to a tightly regulated program. The different parts of the nervous system are built by cell proliferation, migration and differentiation, and proper functioning requires a precise number of cells in the right place with the correct characteristics (Rodier, 1994). Insults interfering with these mechanisms can consequently result in adverse changes in the nervous system. For instance, disruption of proliferation can inhibit the formation of subpopulations of neurons that were forming at the specific time point of the insult. Migration is even more sensitive, since it can be disrupted either directly or by effects on neighbouring cells or important supporting structures, e.g. radial glia. Moreover, alterations in the programmed cell death process, which is regulated by growth factors, cytokines and neurotransmitters (Henderson, 1996; Ikonomidou et al., 2001; Johnson and Deckwerth, 1993), can kill neurons that should not have been removed. They can also promote the survival of the redundant cells that are produced during neurogenesis to ensure the correct formation of a given structure, but under normal conditions are eliminated afterwards. Not only neuronal cells can be affected by developmental injuries. The brain growth spurt, which is the period when the brain grows most rapidly, is predominantly characterized by the proliferation of astrocytes and oligodendrocytes, glial cells that are responsible for a variety of functions, such as neuronal support and maintenance, and myelin production. This stage of development has been shown to be very sensitive to toxic insults, suggesting that glial cells also are a target for neurotoxicants (see Aschner and Allen, 2000). Interestingly, the consequences of a developmental damage may not necessarily be apparent until a critical age when a neurodevelopmental defect may be unmasked or precipitated by a subsequent insult (see Reuhl, 1991).

During this modern era the number of new chemicals appearing annually has increased enormously. Exposure to toxic substances before or after birth has been identified as

one key risk factor for neurodevelopmental disorders, including autism, dyslexia, attention-deficit hyperactivity disorder, decreased intelligence and mental retardation (Grandjean and Landrigan, 2006). Consequently, the need for developmental neurotoxicity studies of environmental/industrial chemicals has been deemed to be of outmost importance. Due to the vast number of chemicals, the amount of animals needed for safety evaluation has substantially increased. Concerning animal use, reproductive and developmental toxicity testing is especially demanding, since at least two generations of animals are involved. Progress in mechanistic research and the increasing awareness of the need to reduce, replace and refine animal testing has led to the development of alternative methods. These *in vitro* methods have been applied in many research fields such as cancer biology, drug discovery, and toxicology. The use of different cell culture models for predicting *in vivo* effects of single neurotoxic chemicals are developed to provide rapid screening systems with a battery of highly sensitive assays. Although alternative testing will not be able to give the extent of information that can be retrieved from animal testing, *in vitro* systems may have a screening function prior to *in vivo* testing. Additionally, early effects at the molecular level, only detectable *in vitro*, can predict toxic effects that would not appear *in vivo* until late development or in the adult. As mentioned above, embryonic development is a continuous process of a precisely orchestrated sequence of events, including cell proliferation, migration, differentiation, and maturation, driven by gene expression changes that are programmed both in time and space. Toxic interference with these programs will most likely give rise to malformations and/or malfunctions. NSCs appear already during the neural plate formation, and is generally agreed to exist throughout the various developmental stages. It has been suggested that NSCs in fact constitute the major cell type of the early ectoderm. Hence, for studies with focus on cellular mechanisms of toxic effects during brain development NSCs are an ideal *in vitro* model. With increased knowledge of mechanisms and the identification of readily measured endpoints, it should be possible to identify patterns, i.e. unique signatures, for different classes of neurotoxicants.

Oxidative stress

Oxidative stress occurs as a consequence of disturbance in the balance between the generation of reactive oxygen species (ROS) and the antioxidant defence mechanisms (Betteridge, 2000; Sies and Cadenas, 1985). ROS have the ability to react with all biological macromolecules, such as lipids, proteins, nucleotides and carbohydrates. Particularly susceptible are polyunsaturated fatty acids, key components of cellular, mitochondrial and nuclear membranes. Excessive amounts of ROS can thus lead to disruption of the cellular integrity (Jaeschke, 1995). It has been suggested that the mitochondrial shutdown seen after heavy oxidative stress could initiate apoptosis and/or necrosis due to the dramatic decrease in cellular energy, and the release of pro-apoptotic factors. For example, nitric oxide can induce apoptotic cell death in NSCs via activation of p38 and MAPK prior to mitochondrial dysfunction and caspase activation, which can be attenuated by Bcl-2 overexpression (Cheng et al., 2001). The

oxidation and modification of the sulfhydryl groups in proteins can alter their normal function (Stadtman, 1993) and ROS-interactions with DNA can cause single-strand breaks and crosslinking, which can lead to PARP-activation (Schraufstatter et al., 1986). Oxidative stress and the oxidative modifications of biomolecules have been reported to play a key role in several physiological and pathophysiological processes, including aging, atherosclerosis, inflammation, cancer, diabetes, Alzheimer's disease, Parkinson's disease, and in response to radiation and toxic chemicals, (Becker et al., 1991; Byczkowski and Gessner, 1988; Carney et al., 1991; Djordjevic et al., 2004).

In aerobic cells the main site for the generation of ROS, such as the superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide, is the mitochondria (Buttke and Sandstrom, 1995; Morel and Barouki, 1999). Here ROS are produced consistently as a by-product of complex I (NADH/ubiquinone oxidoreductase) and complex III (ubiquinol/cytochrome *c* oxidoreductase) activity during mitochondrial respiration. It is estimated that up to 2% of the oxygen reacting in the respiratory chain causes the formation of superoxide radicals. This common ROS can be dismutated into hydrogen peroxide, which itself can be transformed into a more reactive ROS, the hydroxyl radical, by Fenton reaction catalyzed by metal ions (Cu^{2+} and Fe^{2+}) (Djordjevic, 2004). However, it is crucial to understand that ROS are only harmful when oxidative stress is induced. Accumulating data shows that ROS, at physiological levels, may act as an essential second messenger in signal transduction pathways (Suzuki et al., 1997). For example, the small G-protein Ras is believed to activate a cascade of kinases via ROS production (Pennisi, 1997). In addition several transcription factors, such as NFκB, p53 and AP-1, have been shown to be modulated by oxygen species (Morel and Barouki, 1999). It is also generally established that superoxide radicals produced by neutrophiles and other phagocytic cells are a part of the immunological defence against bacteria (Babior, 1978a; Babior, 1978b). Under normal conditions, aerobic cells are capable of neutralizing the small amount of continuously formed ROS. These biochemical antioxidant defenses are mostly present in the mitochondria, and include glutathione, glutathione peroxidase, superoxide dismutase, NADP dehydrogenase, vitamins E and C (Halliwell and Gutteridge, 1988; Kehrer and Lund, 1994; McGowan et al., 1996; Sato et al., 1995). An example of how this system works is the superoxide dismutase scavenging of superoxide radicals into hydrogen peroxidase, which is detoxified into water and oxygen by glutathione peroxidase or catalase.

Staurosporine

Staurosporine (STS), an indolo[2,3-alpha] carbazole (figure 3), was discovered 30 years ago in the course of screening extracts of the bacterium *Streptomyces staurosporeus* for constituent alkaloids with protein kinase C-inhibitory properties for potential modifiers of malignant growth (Omura et al., 1977). STS has since been discovered to have biological activities ranging from anti-fungal to anti-hypertensive (Omura et al., 1995), and to be a broad spectrum protein kinase inhibitor by

preventing ATP-binding to the kinase catalytic domain. Although STS inhibits several protein kinases, including PKA (IC_{50} = 15 nM), PKG (18 nM), CaMKII (20 nM), and MLCK (21 nM), it has the highest affinity to the 12 known protein kinase C isoenzymes (IC_{50} = 2.7 nM) (Meggio et al., 1995; Tamaoki et al., 1986). Its broad activity spectrum renders STS ineffective as an anti-cancer drug due to interference with normal cell processes. However, studies have shown that STS differs from most chemotherapeutic drugs and death-inducing ligands in that it induces cell death in tumour cells normally resistant to these agents (Belmokhtar et al., 2001; Stepczynska et al., 2001; Xue et al., 2003). Thus, extensive research is ongoing to mimic the actions of STS and to produce structurally-derived compounds that are more selective and have fewer side effects.

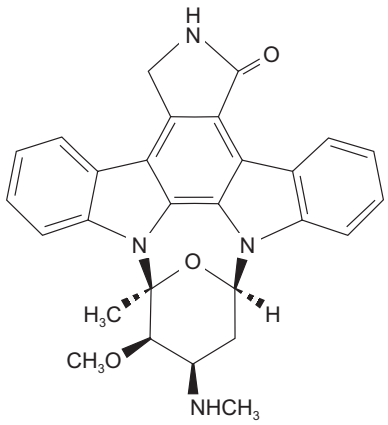

Figure 3. Structure formula for STS

Instead, STS has long been used *in vitro* as an initiator of apoptotic cell death in many different cell types. However, the mechanisms by which STS induce apoptosis remains hard to define. STS has been shown to inhibit the serine/threonine kinase Akt/PKB, leading to decreased phosphorylation of Bad (Franke and Cantley, 1997; Zha et al., 1996). Phosphorylated Bad cannot bind to and antagonize the anti-apoptotic actions of either Bcl-X_L or Bcl-2. Thus, an inhibition of Akt-mediated phosphorylation of Bad would increase sensitivity of cells to apoptosis. Concurringly, STS have been shown to induce the release of cytochrome *c* (Krohn et al., 1998) and caspase activation (Krohn et al., 1998; Krohn et al., 1999). Although it is generally believed that the mitochondrial pathway plays a critical role in STS-induced apoptosis, other studies have shown that Bcl-2 overexpression was ineffective in protecting cells from STS (Yuste et al., 2002) and caspase-independent mechanisms has been suggested (Belmokhtar et al., 2001; Xue et al., 2003). In addition, STS can instigate intracellular ROS accumulation (Krohn et al., 1998; Kruman et al., 1998; Prehn et al., 1997), and increase intracellular Ca^{2+} (Kruman et al., 1998). To boot, antioxidant pre-treatment can prevent STS-induced intracellular Ca^{2+} increase, caspase-3 like activity, DNA- fragmentation, and cell death (Gil et al., 2003).

Furthermore, previous work has shown that STS is able to induce neurite outgrowth in murine neuroblastoma cell lines (Leli et al., 1993; Lombet et al., 2001; Sano et al., 1994). Unlike the neurite outgrowths induced by NGF, those formed in response to STS were reduced in length and did not form neurite networks (Rasouly et al., 1992). In contrast, (Schumacher et al., 2003) demonstrated that low doses of STS generated extremely long axon-like neurites and extensive neuronal networks in embryonic stem cell (ES) cultures. They further demonstrated that STS-treated ES cell lines possess the biological properties of EGF-responsive, undifferentiated neural precursor cells and could be differentiated in high percentage to neuronal and glial cells.

DMNQ

Quinones are widely distributed in nature and can be found in nearly all respiring animal and plant cells. Their vast redox potential is mainly used for transporting electrons from one substance to another in enzyme-catalyzed reactions, and they play vital physiological parts in a number of processes, such as the photosynthesis and the respiratory chain in the mitochondria. In nature these compounds are often referred to as ubiquinones (*ubi*quitous *quinone*) or coenzymes Q. Some quinones are also used as anticancer, antimalarial, or antibacterial drugs (Lown, 1983; Powis, 1987; Vennerstrom and Eaton, 1988). However, their therapeutic use is limited because of the adverse side effects derived from their cytotoxicity. The toxicity of quinones has been ascribed to two main mechanisms. The first is the arylation of nucleophiles among critical cellular proteins and/or DNA. For instance, quinones react covalently with thiols, such as glutathione (GSH) or the cysteine residues of proteins, to form arylation products that eventually cause cellular damage (Tapper et al., 2000). The second mechanism is the induction of oxidative stress via redox cycling (figure 4). One-electron reduction by e.g. NADPH cytochrome P450 reductase, NADH-cytochrome b_5 reductase, and all three nitric oxide synthase (Garner et al., 1999; Matsuda et al., 2000), yield semiquinone radicals. These radicals can be reoxidized and thus enter redox cycles with molecular oxygen to form superoxide anions and regenerated quinones (Kappus and Sies, 1981). The two-electron reduction of quinones, catalyzed by e.g. NAD(P)H quinone oxidoreductase, instead yield a much less reactive hydroquinone.

Naphthoquinones consists of naphthalene rings with two ketone moieties in any position, and can be substituted in all positions but the ketone groups. Naphthoquinone derivatives are known to possess anti-bacterial and anti-tumor properties. The use of naphthoquinoid compounds as free radical initiators is often compromised by their propensity to undergo nucleophilic alkylation as mentioned

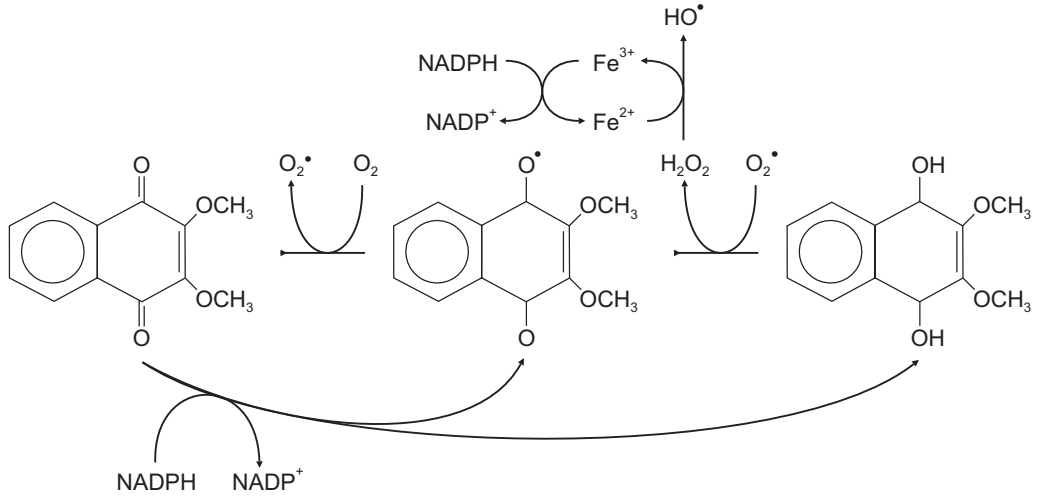

Figure 4. Reaction scheme of DMNQ redox cycling. Reducing agents like NAD(P)H provide the electrons to reduce the quinone moiety and sustain the cycle, continuously reducing oxygen and producing hydrogen peroxide, superoxide, and hydroxyl radicals.

above. 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) is a non-alkylating, -thiol, or -adduct forming α-naphthoquinone derivative that are used to eliminate the previous mechanistic ambiguity involving redox-cycling quinones. Studies in hepatocytes have established that DMNQ, which do not possess any arylating moieties, exerts its toxicity solely by ROS formation via one-electron-based redox cycling (Gant et al., 1988), and is therefore a valuable tool for the generation of ROS in order to study the role of oxidative stress in cell toxicity, apoptosis and necrosis. Although studies have shown that the arylating mechanism is critical for quinone toxicity (Henry and Wallace, 1996; Seung et al., 1998; Toxopeus et al., 1993), there are recent studies that show that the toxicity of distinct redox cycling quinones is comparable to that of the arylating quinones (Ishihara et al., 2006). For the last decade DMNQ has been used in a number of studies in which the focus has been on studying the effect of oxidative stress in different types of *in vitro* models (Duncan et al., 2003; Ishihara et al., 2006; Shi et al., 1994; Sugawara et al., 2002; von Knethen et al., 1999).

Methylmercury

That mercury is toxic has been known since the ancient times (Clarkson, 1972). Inorganic mercury exists naturally in the earth's crust and is widespread in the environment, but it is also released via anthropogenic sources. In aquatic environments widespread methanogenic bacteria methylate mercury and convert it to methylmercury (MeHg). When MeHg enters living organisms it is passed up the food chain, and the highest levels accumulate in predatory fish and large sea mammals (figure 5). Dietary MeHg is almost totally absorbed in the gastrointestinal tract and rapidly enters the bloodstream. It can easily cross the blood-brain barrier and therefore makes the brain a primary target organ (Clarkson, 1997). In pregnant women, MeHg readily crosses the placenta and has a high affinity for fetal hemoglobin. Levels in fetal blood are about 25% higher than in the mother (Amin-Zaki et al., 1976). The specific neurotoxicity of MeHg tragically manifested itself in catastrophes in Japan and Iraq, where large populations were exposed to high-levels of MeHg (Bakir et al., 1973; Takeuchi et al., 1959). In Japan a factory producing acetaldehyde accidentally released MeHg directly into the water. Since MeHg accumulates in the aquatic food chain, the fish eating population was exposed to exceptionally high amounts. In Iraq numerous people was exposed to high concentrations of MeHg in the beginning of the 1970's when MeHg-contaminated grain was used for bread baking. Both prenatal and adult exposure to MeHg causes severe CNS damage (Aschner and Aschner, 1990; Clarkson, 1997; Johansson et al., 2007), but it has been shown that the susceptibility is higher during development when fetuses exposed *in utero* can be affected in the absence of maternal toxicity (Choi et al., 1978; Matsumoto et al., 1965; Takeuchi, 1982). Following developmental exposure in mammals, cell loss in the cerebellum and the cerebrum, atrophic brains, delayed and/or abnormal development of the cerebral granule cell layer, atypical cortical migration and cortical disorganization can be seen (Burbacher et al., 1990; Chang, 1977; Eto et al., 1997). However, the pattern of damage in both

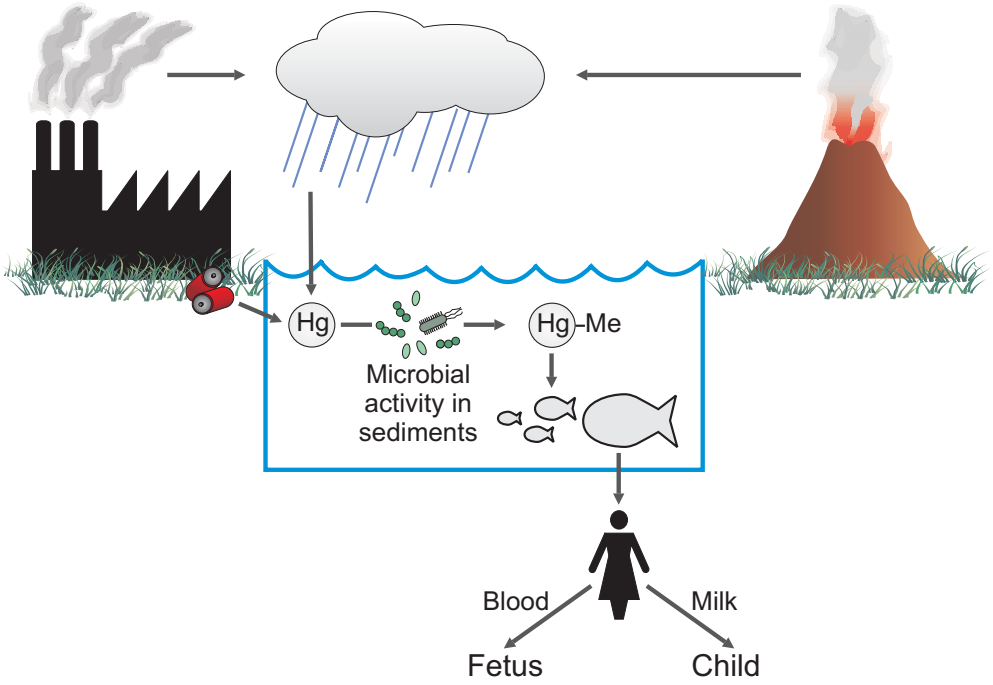

Figure 5. A simplified illustration of the overall process of mercury release in the environment. Mercury is methylated into methylmercury and, through bio-magnification, accumulates in the aquatic food chain. Thus, fish, shellfish and sea mammals are the main source of human exposure to methylmercury.

the fetus and infant, unlike in adults, is less defined and seems to depend on concentration and duration as well as the stage of development when the exposure occurs (Rodier, 1995). At cellular level the cytotoxicity of MeHg has been ascribed to three major mechanisms: i) perturbation of intracellular Ca^{2+} levels (Atchison and Hare, 1994; Graff et al., 1997; Sarafian, 1993); ii) induction of oxidative stress by either overproduction of ROS (LeBel et al., 1990; Sarafian and Verity, 1991) or by reduced oxidative defense (LeBel et al., 1990; Sarafian and Verity, 1991; Yee and Choi, 1994); and iii) interactions with sulphydryl groups and thus forming complexes with thiol-containing compounds (Clarkson, 1972) targeting proteins and peptides containing cysteine and methionine.

Manganese

Rock weathering and wind erosion cause release of manganese (Mn) into the surrounding environment. This natural redistribution of manganese is generally more important for the manganese concentration in soil, plants, water and air than manganese from anthropogenic sources. Mn is an essential trace element for all living organisms where it plays an important role in various parts of the metabolism. Manganese is required for normal amino acid, lipid, protein, and carbohydrate metabolism and is needed by the fetus to support normal growth and development (Dorman et al., 2006). Mn is utilized by various antioxidant enzymes such as superoxide dismutase and glutamine synthase (Cotzias, 1958; Takeda, 2003; Wedler and Denman, 1984). Changes in Mn-containing proteins have been observed in several neurodegenerative disorders, such as Alzheimer's disease and amyotrophic

lateral sclerosis. In addition, chronic exposure to high levels of Mn has been associated with severe extrapyramidal dysfunction referred to as manganism resembling the dystonic movements seen in Parkinson's disease. However, several clinical features are distinct from Idiopathic Parkinson's disease, and patients suffering from manganism do not respond to dopamine replacement (Huang et al., 1993; Markesbery, 1997; Olanow et al., 1996; Pal et al., 1999).

Manganese deficiency, characterized by weight loss and blood clotting problems, is rare since Mn is a ubiquitous element found in many foods. Hence, in humans pathologies associated with abnormal Mn biology are mainly associated with exposure to excessive amounts of Mn. Most cases of manganism stem form occupational exposure from for example ore mining, ferroalloy plants, and the battery industry (Lucchini et al., 1999; Mena et al., 1970). In the non-occupational environment ingestion of manganese through food is the major exposure route. Ingestion of water usually constitutes a minor exposure route for humans. However, it is recognized as an important source of exposure in newborns receiving infant formula (Sievers, 2005). Adults usually maintain a stable manganese tissue level due to that the gastrointestinal absorption as well as the hepatobiliary excretion are strictly regulated (Aschner et al., 2005; Dorman et al., 2006).

The main target for manganese toxicity is the CNS, but organs such as liver and lung as well as the reproductive and immune system are affected (Misselwitz et al., 1995). Manganese can be transported across the blood brain barrier via both active and passive mechanisms, including facilitated diffusion, active transport, DMT-1 mediated transport, ZIP8 transport, and store-operated calcium channels as well as transferrin dependent transport (Erikson et al., 2007). Manganese is eliminated from the brain over time with a half-life of 50-75 days in rodents and non-human primates, which is longer than for the rest of the body (Dorman et al., 2006). As a divalent cation, manganese is able to bind to negatively charged ions such as nitrate, phosphate and carboxylate groups. It is thereby able to react with proteins and can inhibit or activate various enzymes. Generally Mn is alleged to exert cellular toxicity via a number of mechanisms, including a direct or indirect formation of ROS (Ali et al., 1995; Brouillet et al., 1993), the direct oxidation of biological molecules (Archibald and Tyree, 1987), disruption of the mitochondrial energy production (Zheng et al., 1998), and the disturbance of Ca^{2+} and iron homeostasis (Gavin et al., 1990; Kwik-Uribe et al., 2003; Zheng and Zhao, 2001; Zheng et al., 1999).

That Mn overexposure may have negative developmental effects has been proposed since the late 1970's, and recent epidemiologic studies by Wasserman and collegues showed that Bangladeshi children who drank well water with high concentrations of Mn had decreased intellectual function (Wasserman et al., 2006). In addition, studies have shown an increased brain accumulation of Mn and more pronounced brain pathology in neonatal rats compared to adults after exposure to similar doses, which

probably can be ascribed to a more vulnerable nervous system, an incomplete blood-brain barrier, lower bile excretion, and the not yet fully developed gastrointestinal absorption homeostasis (Chandra and Shukla, 1978; Dorman et al., 2000; Kostial et al., 1978; Miller et al., 1975; Rehnberg et al., 1982; Seth et al., 1977; Shukla et al., 1980).

AIM OF THE STUDY

The specific objectives of this study were:

- To establish relevant *in vitro* experimental models for neurotoxicity studies using neural stem cells.
- To identify the intracellular pathways activated by well established apoptosis-inducers, such as staurosporine and Fas ligand.
- To characterize the type of cell death occurring in neural stem cells exposed to oxidative stress.
- To investigate the effect of the neurotoxic metals MeHg and Mn on neural stem cell survival and differentiation.

MATERIAL AND METHODS

Detailed descriptions of the material and methods used in our studies can be found in the articles and submitted manuscripts included in this thesis.

CELL CULTURE PROCEDURES

In paper I we used a primary culture of aNSCs. Adult rats were sacrificed and the anterior portion of the lateral wall of the lateral ventricles were dissected out and enzymatically dissociated. The cells were cultured in suspension in tissue culture dishes in media consisted of DMEM/F12 with Glutamax supplemented with B27, 8 mM Hepes, 100 U/ml penicillin and 100 µg/ml streptomycin. Twenty ng/ml EGF was added every 48 h. After 6-8 days, primary neurospheres were passaged to generate secondary neurospheres. Trypsinated single cells were cultured in suspension in 50:50 mixtures of fresh and conditioned media. Secondary neurospheres were split again and seeded on poly-$_L$-lysine coated coverslips. Cells were allowed to settle and attach for 1 h before experimental treatment.

Paper II is based on experiments on the C17.2 cell line, a clonal multipotent neural precursor cell line originally derived from the external germinal layer of neonatal mouse cerebellum (Snyder et al., 1992). Cells were cultured as monolayers and routinely seeded at the density of 3000 cells/cm^2 in cell culture dishes and maintained in DMEM supplemented with 10% fetal calf serum (FCS), 5% horse serum, 4 mM L-glutamine, gentamicine, and fungizone in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For experimental analyses, cells were grown in either cell culture dishes or on poly-$_L$-lysine coated coverslips.

In paper III, IV and V we used the C17.2 cell line described above as well as primary cultures of cortical neural stem cells (cNSCs) obtained from E15 rat embryos. The tissue was dissected out and gently mechanically dispersed. The cells were seeded in 100 mm cell culture dishes precoated with poly-$_L$-ornithine and fibronectin. Cells were cultured as monolayers and maintained in DMEM:F12 supplemented with N2 (Bottenstein and Sato, 1979). Ten ng/ml bFGF was added every 24 h and the medium was changed every other day. When subconfluent, cells were passaged and reseeded at desired plating densities. The cells were used for experiments 48 h after the first and second passage.

In paper V, in addition to the C17.2 cell line and the primary cultures of cNSCs, we employed the mouse hippocampal HT22 cell line and the human astrocytoma D384 cell line. The D384 cell line was established from a human astrocytoma (Balmforth et al., 1986). Cells were routinely seeded at the density at 10000 cells/cm^2 in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and cultured at 37°C in humidified air with 5% CO_2. The HT22 cell line, an immortalized

hippocampal cell line, is a subclone of the original HT4 clone (Morimoto and Koshland, 1990). Cells were routinely seeded at a density of 3000 cells/cm^2 in CO_2-independent medium supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell culture flasks were locked and kept at 37°C.

In paper VI, the experiments were performed solely on primary cultures of cNSCs.

At the time of experiments NSCs were nestin-positive, confirming their proliferative and undifferentiated status. In addition, all three NSC models have been shown to be able to differentiate into neurons, astrocytes and oligodendrocytes (Johe et al., 1996; Reynolds et al., 1992; Ryder et al., 1990).

ANIMALS

For the preparations of aNSCs, two adult (3 months old) Sprague-Dawley rats were housed together and checked upon twice daily, for each primary culture preparation. Animals were kept in air-conditioned quarters, with controlled daylight cycles and free access to food and water. All procedures used during the animal experiments comply with Karolinska Institutet's guidelines in the care and use of laboratory animals. For preparation of cNSCs one time-pregnant Sprague-Dawley rat was used for each primary culture preparation. The animal was kept in a similar manner, and the same guidelines as mentioned above were followed.

EXPOSURE PROCEDURES

In the following studies we have exposed our different stem cell models to various toxicants. In paper I we used STS, an inhibitor of protein kinase C and a known inducer of apoptosis (Tamaoki et al., 1986), to identify key players in the apoptotic machinery in aNSCs. To induce apoptosis via the Fas receptor, cells were exposed to Fas mAb, which have been shown to induce apoptosis in other cell models (Feng and Kaplowitz, 2000). STS and Fas mAb were also used in paper II, in which we focused our studies on the C17.2 cell line. Oxidative stress was experimentally induced by adding 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), which exerts is toxic properties via redox cycling activity (Gant et al., 1988; Henry and Wallace, 1995). Actinomycin D and cycloheximide were employed to block mRNA and protein synthesis, respectively. The general caspase inhibitor zVAD-fmk or the antioxidant Mn(III)tetrakis(4-benzoic acid) porphyrin (MnTBAP) treatments were administered 1 h prior to DMNQ-exposure. In paper III we exposed C17.2 cells and cNSCs to MeHg. Caspases and calpains were inhibited zVAD-fmk and E64d, respectively. In paper IV we again exposed C17.2 cells as well as the cNSCs to DMNQ. Caspase-2 and caspase-3 were inhibited specifically with most selective inhibitors zVDVAD-fmk and zDEVD-fmk, respectively. Antioxidant treatment with Trolox or caspase inhibition took place 1 h prior to toxicant exposure. In paper V we exposed the C17.2, HT22, D384 cell lines as well as cNSCs to $MnCl_2$. The selective caspase-8 inhibitor

zIETD-fmk and the specific p38 MAPK inhibitor SB203580, as well as zVAD-fmk and MnTBAP, were administrered 1h prior to $MnCl_2$ exposure. In paper VI cNSCs were exposed to MeHg and $MnCl_2$. The metalloprotease inhibitor GM6001 was administered 1 h before cells were exposed to the toxicant. All treatments were administered by direct dilution into the culture medium and an equivalent volume of vehicle was added to the control cultures.

MTT ASSAY

This assay is useful for determining cell survival and function of mitochondrial enzymes (Ankarcrona et al., 1995; Mosmann, 1983). It is based on the capacity of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), yielding purple formazan crystals. One hour before the end of exposure, 0.5 mg/ml MTT was added to the samples. At the end of the incubation, the crystals were dissolved by the addition of a solubilization solution (0.04 M HCl in anhydrous isopropanol). The culture plates were then shaken to ensure that all the formazan crystals were dissolved, and aliquots of solution were pipetted into a 96 well microplate. The absorbance of the resulting purple solution was measured at a wavelength of 570 nm using a multiwell spectrophotometer.

TRYPAN BLUE EXCLUSION TEST

To distinguish cells undergoing necrotic cell death, cells were trypisinzed after treatment and the cell suspension was mixed with an equal amount of 0.4 % Trypan blue solution. Using a Neubauer counting chamber cells were counted under a phase contrast microscope. Cells with a damaged cell membrane (necrotic cells) stained blue, while cells with intact plasma membrane (healthy or apoptotic cells) remained unstained.

NUCLEAR STAINING WITH HOECHST 33342 AND PROPIDIUM IODIDE

To evaluate the nuclear morphology, cells were grown on coverslips and fixed with either 80% ice cold methanol at -20°C or 4% paraformaldehyde at 4°C. After rinsing, cells were stained with propidium iodide (PI) or Hoechst 33342. The coverslips were subsequently mounted with glycerol-PBS containing phenylenediamine and examined using a fluorescent microscope. Cells were counted, scoring at least 300 cells in 5 microscopic fields randomly selected on each coverslip. The percentage of apoptotic cells was determined by counting the amount of cells exhibiting apoptotic chromatin condensation compared to the total amount of cells. Cells were examined using an Olympus BX60 fluorescence microscope equipped with a C4742-95-10sc digital camera.

ANNEXIN-V LABELLING

To detect and visualize the translocation of phosphatidylserine (PS) from the internal to the external surface of plasma membrane, a hallmark for apoptosis, cells cultured on coverslips were washed with a binding buffer (0.01 M HEPES, 0.01 M NaOH, 0.14 M NaCl, 2.5 mM $CaCl_2$) and incubated with FITC-conjugated Annexin-V, which binds to PS. Cells were concurrently stained with PI and Hoechst 33342. Coverslips were mounted with binding buffer and analyzed under fluorescence microscope. When using non-fixed cells, this method makes it possible to assess necrosis and apoptosis at the same time. An intact plasma membrane is non-permeable for PI. Consequently necrotic cells stain positive for all three dyes, whereas apoptotic cells only stain positive for Hoechst and Annexin-V. Cells were examined using an Olympus BX60 fluorescence microscope equipped with a C4742-95-10sc digital camera.

MEASUREMENT OF CASPASE ACTIVITY

To evaluate the activation of caspases we measured the cleavage activity on caspase-2, -3, -8 and -9 specific fluorogenic peptide substrates: VDVAD-AMC, DEVD-AMC, IETD-AMC, and LEHD-AMC, respectively. The measurement was performed using a fluorometric assay reported previously (Nicholson et al., 1995), with some modifications (Gorman et al., 1999). After exposure to various treatments cells were scraped gently from the plates, collected in tubes and washed with PBS. Cells were transferred to separate wells of a 96-well plate and frozen on liquid nitrogen. For DEVD-AMC and LEHD-AMC cleavage, cells were resuspended in PBS, added to a microtiter plate, and combined with substrate dissolved in a standard reaction buffer (100 mM Hepes, pH 7.25, 10% sucrose, 10 mM dithiothreitol (DTT), 0.1% CHAPS). For VDVAD-AMC or IETD-AMC cleavage, cells were resuspended in PBS, added to a microtiter plate, and combined with substrate dissolved in a standard reaction buffer (100 mM MES, pH 6.5, 10% polyethylene glycol, 10 mM DTT, 0.1% CHAPS). Substrate cleavage leading to the release of free AMC was monitored at 37°C using a Fluoroskan II (excitation 355 nm, emission 460 nm). Fluorescent units were converted to pmoles of AMC and subsequently related to the amount of proteins in each sample.

IMMUNOCYTOCHEMISTRY

The detection of polypeptides with immunocytochemistry offers an opportunity to analyse cell mechanisms at single cell level. Cells were grown on coverslips, treated, subsequently fixed with either 4% paraformaldehyde or 80% methanol, and then washed with PBS. Primary antibodies (see table 1) were diluted in PBS with 0.3% Triton-X100 and 0.5% BSA. Cells were incubated with primary antibodies in a humid chamber at 4°C overnight, washed with PBS and incubated with the appropriate secondary fluorophore-conjugated antibodies. After rinsing with PBS, during which cells were co-stained with Hoechst 33342, coverslips were mounted in glycerol-PBS containing phenylenediamine or Vectashield® mounting media. Cells

were examined using an Olympus BX60 fluorescence microscope equipped with a C4742-95-10sc digital camera, or a Zeiss LSM 510 Meta confocal microscope.

Table 1. Primary antibodies used for immunocytochemical staining

Antigen	Raised in	Dilution	Source
AIF	Goat	1:200	Santa Cruz
Bax	Rabbit	1:400	Santa Cruz
Bax	Rabbit	1:400	BD Pharmingen
Caspase-3 (active)	Rabbit	1:300	Kouroku et al, 2000
Caspase-3 (active)	Rabbit	1:50	Cell signaling
Caspase-8	Rabbit	1:500	BD Pharmingen
Caspase-9 (active)	Rabbit	1:400	Kouroku et al, 2000
CD95 (Fas/Apo-1)	Rat	1:200	Med. & Biol. lab.
Cytochrome *c*	Mouse	1:100	BD Pharmingen
GFAP	Rabbit	1:300	Dako
Nestin	Rabbit	1:1000	Dahlstrand et al, 2002
βIII-tubulin	Mouse	1:400	Nordic Biosite

PULSE FIELD GEL ELECTROPHORESIS

Degradation of chromatin into large size DNA fragments, another hallmark of apoptosis, was measured by field inversion gel electrophoresis (FIGE) described previously (Zhivotovsky et al., 1994). Briefly, after treatment cells were gently harvested and washed with PBS. Cells were immobilized in 1:1 mixture of buffer (150 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 2 mM KH_2PO_4-KOH pH 6.8) and 1% low-melting point agarose into gel plugs. After solidification, the plugs were subjected to proteinase K digestion prior to loading into agarose gels. The DNA was separated by FIGE at constant voltage (180 V) using a switchback pulse controller. The ramping rate changed from 0.8 s to 30 s over the 24 h, with a forward to reverse ratio of 3:1. Two sets of pulse markers DNA were used for size: i) chromosomes from *Saccharomyces cerevisiae* (225-2200 kbp); and ii) a mixture of λ-DNA, λ-Hind III fragments, and λ-DNA concatemers (0.1-200 kbp). The gels were stained with ethidium bromide to visualize the DNA.

TUNEL STAINING

Induction of single strand breaks of DNA after toxic exposure was determined by using terminal deoxyribonucleotide transferase [TdT]-mediated dUDP nick end labeling (TUNEL), a method allowing the identification of DNA fragments with 3'-hydroxyl ends by fluorescent labeling. Cells were grown on coverslips and were fixed with 4% paraformaldehyde after treatment. Cells were stained with a reaction mixture containing 0.1% Triton, 5x terminal transferase buffer, 25 μM $CoCl_2$, 1 mM fluorescein-12-UTP, and 25 U/μl terminal transferase. After incubation at 37°C, the coverslips were mounted with glycerol-PBS containing phenylenediamine and examined with a fluorescent microscope.

WESTERN BLOT ANALYSIS

Standard Western blotting procedure was used for the immunodetection of proteins. After treatment, cells were harvested by trypsination, washed with PBS and incubated

in lysis buffer (10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP40, 0.1 mM PMSF, 1 mM DTT, 2 µg/ml aprotinin). For fractionation of organelles and cytosol, cells were harvested and incubated in 0.005 % digitonin buffer (250 mM sucrose, 20 mM Hepes pH 7.4, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, and 1 mM EGTA). Supernatant fraction, containing cytosolic proteins, and pelleted membrane fraction, containing mitochondria, were obtained via centrifugation. Total protein content of samples was determined with Micro BCA protein assay kit to ensure equal protein loading at gel electrophoresis. Samples were subjected to SDS-PAGE, followed by electroblotting to nitrocellulose membranes. Membranes were probed overnight with primary antibodies (see table 2). The membranes were rinsed, incubated with appropriate horseradish peroxidase-conjugated secondary antibodies, and developed with ECL reagents for chemiluminescence and exposed to X-ray autoradiography films. Equal protein loading was confirmed by re-probing the membranes with rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 2. Primary antibodies used for immunoblotting

Antigen	Raised in	Dilution	Source
Bax	Mouse	1:1000	BD Pharmingen
Bid	Rabbit	1:300	BD Pharmingen
Caspase-8	Rabbit	1:500	BD Pharmingen
CD95 (Fas/Apo-1) (M20)	Rabbit	1:400	Santa Cruz
Cytochrome *c*	Mouse	1:2500	BD Pharmingen
Fodrin	Mouse	1:1000	Chemicon
GAPDH	Rabbit	1:3000	Trevigen
GAPDH	Rabbit	1:1000	Nordic Biosite
NICD	Rabbit	1:1000	Cell signaling
p53	Rabbit	1:1000	R&D Systems
PARP	Mouse	1:1000	Santa Cruz
Phospho-p42/44	Rabbit	1:1000	In vitro

ATP DETERMINATION

Mitochondrial ATP synthesis is mainly driven by membrane potential generated by electron transfer via the respiratory chain and the subsequent pumping of protons from the mitochondrial matrix. While apoptosis is an energy-dependent process, one evident physiological difference in cells undergoing necrosis is the depletion of intracellular ATP. Thus, fluxes in intracellular ATP levels may switch the decision between apoptosis and necrosis (Eguchi et al., 1997). ATP levels were determined according to the instructions for the ATP Bioluminescence Assay Kit CLS II. Briefly, one million cells were collected and mixed with boiling buffer (100 mM Tris, 4 mM EDTA, pH 7.75) and further boiled for 2 min. The suspension was then centrifuged and ATP was measured in the supernatant by adding an equal volume of luciferase reagent. Sample ATP levels were rectified against a standard curve.

MEASUREMENTS OF MITOCHONDRIAL CA^{2+} UPTAKE RATE

Sequestering of cytosolic Ca^{2+} is an important mitochondrial function, which is also supported by the mitochondrial membrane potential. The capacity and rate of mitochondrial Ca^{2+} uptake reflect the stability of the mitochondria. For measurement

of the mitochondrial sequestering rate of Ca^{2+}, one million cells collected, washed with PBS, and suspended in 400 µl of buffer (150 mM KCl, 5 mM KH_2PO_4, 5 mM succinate, 1 mM $MgSO_4$, 5 mM Tris, pH 7.4). Cells were permeabilized with 0.005% digitonin and 2 µM rotenone was added in order to maintain pyridine nucleotides in a reduced form. Mitochondrial calcium uptake was induced by sequential additions of calcium to the cells. Calcium concentration changes were registered using a calcium-sensitive electrode and visualized with a chart recorder.

VISUALIZATION OF REACTIVE OXYGEN SPECIES IN LIVE CELLS

The Image-IT™ live green ROS detection system was used to visualize ROS in non-fixed NSCs. This assay approach is based on 5-(and-6)-carboxy-2',7'-dichloro-dihydrofluorescein diacetate (carboxy-H2DCFDA) (Armeni et al., 2004; Minami et al., 2005). The non-fluorescent carboxy-H2DCFDA enters live cells and is deacetylated by non-specific intracellular esterases. In the presence of ROS, the reduced fluorescein compound is oxidized and emits bright green fluorescence.

NUCLEOFECTION DELIVERY OF NOTCH REPORTER CONSTRUCT

To investigate the activation of Notch we used a nucleofection strategy for efficient gene delivery of a fluorescent-based reporter construct driven by a promotor with a highly specific response element composed of 12 multimerized binding motifs for the Notch-activated transcription factor CSL. Primary cultured cNSCs were collected and resuspended in Nucleofector solution and transferred to an electroporation cuvette. The Notch reporter construct 12XCSL-DsRedExpressDR was added to the cell suspension as well as a pmaxGFP-construct, and transfection was performed using the Amaxa Biosystems Nucleofector device. Nucleofected cells were seeded on polyornithine and fibronectin precoated coverslips, and kept with 10 ng/ml bFGF for 24 h before the start of experiments at which time point the FGF was removed.

DIFFERENTIATION ASSAY

Primary cultures of cNSCs were plated at a low density on coverslips coated with poly-$_L$-ornithine and fibronectin, and grown in the presence of bFGF. Forty-eight hours after the first passage, medium was changed and no further bFGF was added during the course of the experiment to promote spontaneous differentiation. Cells were exposed once to MeHg and $MnCl_2$ for 7 days. Subsequently cells were fixed with 4% paraformaldehyde and incubated with primary antibodies diluted in PBS with 0.3% Triton-X100 and 0.5% BSA. To determine the neuronal differentiation cells were stained with mouse anti βIII-tubulin, and Hoechst 33342. After incubation with the appropriate secondary antibody, coverslips were rinsed and mounted in glycerol-PBS containing phenylenediamine or Vectashield® mounting medium. Positively stained cells were estimated in 5 microscopic fields randomly selected on each coverslip and then related to the total number of cells of each field as assessed by Hoechst 33342 stained nuclei.

RESULTS

PAPER I

In the first paper we used STS, a known inducer of apoptosis and inhibitor of protein kinase C, to identify key players in the apoptotic machinery of primary cultures of rat aNSCs. Primary cultures of dispersed adult rat lateral ventricular walls were grown in cell suspension to form primary neurospheres, which were dissociated and cultured to generate secondary neurospheres. When plated on poly-$_L$-lysine coated coverslips, cells could positively be labelled for the stem cell marker protein nestin. Our experiments showed that aNSCs exposed to STS (0.25 μM) exhibited characteristic morphological hallmarks of apoptosis, such as condensed nuclei and chromatin condensation. We could also detect TUNEL positive cells, indicating the presence of DNA-fragmentation with 3'hydroxyl ends due to activation of specific endonucleases, and translocation of phosphatidylserine to the outer leaf of the plasma membrane. After establishing that STS induced apoptosis in our model system, we next determined the apoptotic pathway and the signaling cascade. The aNSCs with characteristic apoptotic morphology described above also showed active caspase-9 and caspase-3 in the cytoplasm, which both could be inhibited with pan-caspase inhibitor zVAD-fmk pre-treatment. We could also demonstrate the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol, establishing the key role of mitochondria and caspases in the intrinsic apoptotic machinery of aNSCs. When investigating the extrinsic death receptor pathway, we found that NSCs in adult rats expressed the Fas receptor, but that the Fas-activating antibodies did not induce cell death.

PAPER II

In our second paper we used the C17.2 cell line as a model for developmental NSCs and investigated the apoptotic machinery after toxic stimuli, such as oxidative stress as well as STS. Cells were treated with DMNQ, which undergoes redox cycling that produces O_2^- and H_2O_2 and thus causes oxidative stress (Gant et al., 1988; Shi et al., 1994). DMNQ (30 μM) and STS (0.25 μM) both induced DNA-fragmentation and apoptotic morphology in C17.2 cells, which we also saw in aNSCs. Activation of caspases-9 and -3 was also detected, which could be inhibited along with apoptotic morphology after pre-treatment with zVAD-fmk (20 μM). As seen in the aNSCs, the cytotoxic stimuli induced cytochrome *c* release from the intermembrane space of the mitochondria. The activation of caspases, along with the chromatin condensation, could significantly be reduced with pre-treatment with the antioxidant MnTBAP (100 μM), which has been shown to have superoxide dismutase and catalase activities (Day et al., 1995). This confirms that oxidative stress induces apoptosis via a caspase-dependent mechanism in C17.2 cells. Furthermore, we could detect apoptosis inducing factor (AIF) translocation from the mitochondria to the nucleus in the C17.2 cells treated with DMNQ. This result is in agreement with a previous study from our

group (Fonfria et al., 2002), showing AIF translocation in neurons exposed to MeHg or hydrogen peroxide (H_2O_2). These observations support the idea of redundant pathways activated during apoptosis in NSCs. Our studies show that the mitochondrial apoptotic pathway is operative in C17.2 cells, as further supported by data showing that Bcl-2 overexpression inhibits apoptosis in C17.2 cells exposed to nitric oxide (Cheng et al., 2001).

As in the aNSCs, apoptosis could not be induced in C17.2 cells by exposure to Fas mAb (250 ng/ml), and no activation of caspase-8 or caspase-3 could be detected in the exposed cells. We also measured the procaspase-8 levels in the C17.2 cells to determine whether the Fas mAb insensitivity was due to decreased procaspase-8 expression. The results showed that procaspase-8 is abundantly expressed in C17.2 cells. Downregulation of FLIP has been shown to increase sensitivity to Fas (Tschopp et al., 1998). In our experiments, pre-treatment with actinomycin D or cycloheximide, inhibitors of transcription and protein synthesis respectively, did not influence the sensitivity to Fas mAb in C17.2 cells. This suggests that FLIP may not be responsible for the inhibition of the Fas death receptor pathway. It has been shown that Fas can mediate proliferation and regeneration in neuronal systems via the extracellular-signal regulated kinase (ERK) pathway (Desbarats et al., 2003). Therefore, we investigated whether ERK was activated by Fas in C17.2 cells. Short exposure of C17.2 cells to Fas mAb induced an increase in the dual phosphorylation of ERK at Thr 202 and Tyr 204.

PAPER III

In the third paper, we used the murine NSC cell line C17.2 and primary cultures of cNSCs from cortices of E15 rat embryos to investigate the cytotoxic effects of the environmental organometal MeHg. At cellular level, MeHg has been reported to induce oxidative stress by either overproduction of ROS (LeBel et al., 1990; Sarafian and Verity, 1991) or by reducing antioxidant defenses (Sarafian and Verity, 1991; Yee and Choi, 1994). Therefore, we started to investigate the intrinsic mitochondrial pathway, since in paper II we showed that DMNQ-induced oxidative stress caused apoptosis via this pathway. We used similar doses of MeHg (0.5-2 μM), which we have previously used to induce apoptotic cell death in neuronal or glial cells (Dare et al., 2000; Dare et al., 2001b). We could detect cell death in C17.2 cells with typical apoptotic morphology at the lower doses (0.5 μM). At higher doses (>1 μM) we observed significant levels of necrosis. In cNSCs, the same concentrations (0.5-2 μM) induced necrotic plasma membrane damage in almost all exposed cells. In order to keep the focus on the identification of the intracellular pathways leading to apoptotic cell death, we decreased the dose 10 fold to 0.05 μM when exposing the cNSCs. Consequently, it seems that embryonic cNSCs are more sensitive to the toxic effects of MeHg when compared to other cell types found in the brain. In both models MeHg induced oligomerization and activation of Bax, which forms a channel or a membrane pore, and allows the release of apoptogenic factors (Antonsson et al., 2000). The

exposed cells also showed clear cytochrome *c* release from the intramembrane space of the mitochondria into the cytosol, with subsequent activation of caspase-3. When investigating caspase-specific cleavage of the endogenous substrate α-fodrin we also detected significant breakdown products of activated calpains. Pre-treatment with the pan-caspase inhibitor zVAD-fmk (20 μM), which completely inhibited caspase activation, and with the general calpain inhibitor E64d (10 μM), significantly but only partially protected C17.2 cells and cNSCs from MeHg-induced apoptosis. Together the inhibitors almost completely prevented apoptosis in both cell models, thus indicating that two independent pathways, one including the activation of caspases and the other the activation of calpains, function in parallel during MeHg-induced apoptotic cell death. At concentrations that are not cytotoxic but more relevant to human exposure, MeHg affected differentiation of neural stem cells. We observed that exposure to very low doses (2.5-5 nM) of MeHg, comparable to the levels measured in the umbilical cord blood of pregnant women in Sweden (Bjornberg et al., 2005), significantly impaired the neuronal differentiation of cNSCs when allowed to spontaneously differentiate.

PAPER IV

In this study we wanted to investigate the pathways activated by DMNQ-induced oxidative stress in cNSCs and extend our C17.2 study. After 24 h exposure cNSCs exhibited a rounded shrunken shape, and the assessment of condensed nuclei pointed to a dose-dependent induction of apoptotic cell death. ROS formation after DMNQ-exposure was confirmed using the 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate-based assay, which mainly recognizes intracellular H_2O_2, and pre-treatment with the antioxidant Trolox significantly decreased the amount of cells exhibiting apoptotic nuclei morphology. To activate the apoptotic machinery, cNSCs were exposed to DMNQ (3 μM) for 24h, fixed and immunocytochemically stained for various factors known to be involved in apoptotic signaling. In agreement with our results from the C17.2 cell line experiments, DMNQ-exposure of cNSCs induced Bax-activation, cytochrome *c* release from the intermembrane space of the mitochondria, and the subsequent downstream activation of caspase-3. Apoptotic morphology after DMNQ-exposure could be significantly reduced with pre-treatment with the pan-caspase inhibitor zVAD-fmk. Overall, although cNSCs seem to be more sensitive than C17.2 cells to oxidative damage, both cell models undergo apoptosis via similar mitochondrial-mediated mechanisms. DMNQ-generated H_2O_2, in presence of Fenton metals, form the hydroxyl radical $OH^{\bullet-}$, which has been shown to be damaging the DNA (Imlay and Linn, 1988), and can induce single as well as double strand breaks (Cantoni et al., 1996). Studies show that ROS-mediated DNA-damage can cause p53 accumulation in a variety of cell types, e.g. in stem cell-derived dopaminergic neurons upstream of mitochondrial permeabilization, cytochrome *c* release and casapse-3 activation (Dumont et al., 1999; Gansauge et al., 1997; von Harsdorf et al., 1999). Concurringly, we could detect a time dependent p53 accumulation after DMNQ-exposure in both cNSCs and the C17.2 cell line.

Furthermore, pre-treatment with Trolox completely abolished the increase in p53, proving it to be downstream of ROS formation. Several recent publications suggest a role for p53 in caspase-2 activation (Zhivotovsky and Orrenius, 2005), and caspase-2 activity has been observed early under neuronal cell death induced by β-amyloid, a known inducer of free radical formation and oxidative stress (Varadarajan et al., 2000). In agreement, DMNQ-generated oxidative stress induced early caspase-2 activation in C17.2. Caspase-3 activation was significantly reduced by pre-treatment with the caspase-2 specific inhibitor zVDVAD-fmk, which also inhibited mitochondrial cytochrome *c* release, Bid and PARP cleavage, and markedly reduced the amount of apoptotic cells while no effect could be seen on p53 accumulation.

PAPER V

In paper V we used both the murine NSC cell line C17.2 and the primary cultures of NSCs from E15 rat embryos cortices to investigate the cytotoxic effects of Mn. Experimental studies have shown that neonatal rats are more sensitive to Mn neurotoxicity than adults, results that have been ascribed to a more vulnerable nervous system, a not yet fully developed blood-brain barrier, lower bile excretion, and a high gastrointestinal absorption (Kostial et al., 1978; Miller et al., 1975; Rehnberg et al., 1982). To see whether NSCs are more sensitive to Mn-exposure than differentiated cells, we compared the C17.2 cell line with the hippocampal neuronal cell line HT22 and the astrocytoma cell line D384. In contrast to HT22 and D384 cells, C17.2 cells exposed to 100 to 250 μM $MnCl_2$ for 24 h exhibited clear morphological changes, and showed a significant decrease in cell viability with increasing concentrations of $MnCl_2$. Studies on primary cultures of neurons and astrocytes have shown that neurons are more susceptible to Mn-toxicity than astrocytes (Normandin and Hazell, 2002). In agreement, a significant toxic effect on the HT22 cell line could be detected at doses between 0.5 to 1 mM, while at these doses the D384 cells were not affected. Few Mn-exposed C17.2 cells exhibited positive Trypan blue staining suggesting that the observed decrease in viability is not caused by uncontrolled necrotic cell demise. We then investigated whether the mitochondria was affected by Mn exposure. Sequestering of cytosolic Ca^{2+} is an important mitochondrial function, which along with ATP synthesis is dependent on the mitochondrial membrane potential. No significant decrease in ATP-levels or rate of mitochondrial Ca^{2+} uptake could be detected in C17.2 cells exposed to $MnCl_2$ for 24 h. Overall, these data further support that the decrease in cell viability is not caused by necrosis.

$MnCl_2$ exposure in C17.2 cells induced Bax oligomerization, cytochrome *c* release from the intramembrane space of the mitochondria, a significant increase in caspase-3-like activity, and caspase-specific cleavage of PARP. Furthermore, primary cNSCs exposed to 100 and 250 μM $MnCl_2$ also exhibited clear morphological changes after 24 h exposure. Assessment of chromatin condensation showed both a dose- and time-dependent increase of apoptotic nuclei. In agreement with the results from the C17.2 cell line experiments, $MnCl_2$ induced Bax oligomerization, cytochrome *c* release,

activation of caspase-3, and PARP cleavage. On the whole, cNSCs seem to have comparable dose-sensitivity to $MnCl_2$ and undergo apoptosis via similar mechanisms as C17.2 cells. In addition, an increase in ROS formation could be observed following $MnCl_2$ exposure (100 μM for 24 h) compared to unexposed control cells. The dot-like pattern of detected ROS, suggests that the ROS formed after $MnCl_2$ exposure in NSCs are generated in the mitochondria and that oxidative stress is involved in $MnCl_2$ induced toxicity. Pre-treatment with antioxidant MnTBAP and caspase-inhibitor zVAD-fmk did significantly increase cell viability in $MnCl_2$ treated cells.

PAPER VI

In paper III we demonstrated that exposure of NSCs to MeHg at subcytotoxic levels resulted in a significant inhibition of neuronal differentiation, but the underlying molecular mechanism was not elucidated. Thus, in paper VI we investigated whether Notch signaling would be activated by low subcytotoxic nanomolar concentrations of MeHg in primary cultures of cNSCs, and if such putative Notch activation could be involved in the inhibition of neuronal differentiation induced by MeHg. It has previously been shown by us and others that these cells express functional Notch receptor and its components (Hermanson et al., 2002). A reporter construct driven by a promotor with specific response element binding motifs for the Notch-activated transcription factor CSL was delivered into the cNSCs by nucleofection. Twenty four hours after nucleofection, the FGF2 was withdrawn from the cell cultures and the NSCs were exposed to 2.5-10 nM of MeHg and cultured in growth factor-free conditions for 6, 12, and 24 h. An increase of Notch/CSL reporter activity could be seen in all MeHg-exposed cells compared to the non-exposed controls. In contrast to MeHg, $MnCl_2$ had no effect on spontaneous neuronal differentiation of NSCs at subcytotxic levels, and consistent with these observations, no difference in Notch/CSL reporter activity could be detected at any time point (6-24 h) or dose (1-25 μM) in Mn-exposed cNSCs when compared to control cells. An additional sign of Notch activation is the cleavage of the receptor and thus the formation of the activated Notch intracellular domain (NICD). A clear increase in NICD levels, as detected by immunoblotting, could be seen after exposure to MeHg (2.5, 5 and 10 nM) for 12 h after FGF2 withdrawal. No change in NICD levels after $MnCl_2$ exposure could be detected. It has has been reported that mercurial compunds can bind and influence the activity of the metalloproteases that control the primary cleavage of the Notch receptor (Bland and Rand, 2006; Bland et al., 2003; Hurlbut et al., 2007). Thus, we pre-treated the cNSCs for 1 h with the potent metalloprotease inhibitor GM6001 (20 μM) before MeHg exposure. Interestingly, this resulted in a significant decrease of the MeHg-induced inhibition of neuronal formation as assessed by the number of TuJ1-positive neuronal cells.

DISCUSSION

Neural stem cells constitute a major part of the developing brain. During development there is a balance between proliferation, differentiation and cell death of stem cells, which is highly regulated by a variety of factors and therefore very sensitive (Sommer and Rao, 2002). Insults of different origin, including toxicants, may induce modulatory changes with severe consequences on the development and function of the nervous system. In the adult, apoptotic cell death has been described in a variety of acute and chronic neurodegenerative diseases, such as stroke, Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Honig and Rosenberg, 2000; Przedborski et al., 2003). In the present studies we have used aNSCs, cNSCs, and the murine-derived immortalized multipotent NSC cell line C17.2 to investigate the intrinsic and extrinsic apoptotic pathways in NSCs. The results showed that both STS and DMNQ triggered apoptotic cell death, as shown by nuclear shrinkage and chromatin condensation. Further evidence of apoptosis was the PS translocation to the outer layer of the cell plasma membrane. PS is an anionic phospholipid normally sequestered in the inner leaflet of the plasma membrane. Externalization of PS has been shown to occur in a number of cell types undergoing apoptosis, including neurons (Rimon et al., 1997). Studies have shown that PS is used as recognition signals for phagocytic uptake and cell clearance (Ren and Savill, 1998). The presence of activated caspase-3 fragments and the increased caspase activity in response to STS and DMNQ supported earlier reports on caspase-3 playing a major role in apoptosis of neuronal progenitor cells (D'Sa-Eipper and Roth, 2000). One major partaker upstream of caspase activation is the mitochondria. In addition to being crucial for energy production, the mitochondria play a critical role in apoptosis regulation. Our data show that NSCs undergoing apoptosis induced by STS or DMNQ, release cytochrome *c* from the intermembrane space of the mitochondria into the cytoplasm, with the subsequent activation of caspase-9 and -3.

A considerable increase in ROS formation was observed following DMNQ exposure, confirming the induction of oxidative stress. In addition, pre-treatment with antioxidants significantly reduced caspase-3 activity after DMNQ exposure, indirectly confirming that oxidative stress induced apoptosis via a caspase-dependent mechanism in our *in vitro* models. The caspase inhibitor zVAD-fmk only partially prevented apoptosis in the C17.2 cells exposed to oxidative stress, suggesting that additional cell death pathways, such as the AIF mediated-pathway, may also be activated. This is in agreement with previous studies from our group (Fonfria et al., 2002) that showed AIF-translocation from the mitochondria to the nucleus during oxidative stress-induced neuronal apoptosis. Interestingly, studies on NSCs have shown that inhibition of caspase-3 also can affect differentiation in postnatal rats, increasing neuronal formation (Levison et al., 2000). Others have reported that overexpression of Bcl-2 in NSCs not

only protects cells from cell death, but also shifts the differentiation pattern from neurogenesis to gliogenesis (Esdar et al., 2001).

In paper IV we further investigated the upstream mechanism of DMNQ-induced cell death and found that the DMNQ-generated oxidative stress caused accumulation of the tumor suppressor p53, which preceded the apoptotic hallmarks in both the cNSCs and the C17.2 cell line. We also saw that antioxidant pre-treatment, which as mentioned above were shown to prevent cell death, also inhibited p53 accumulation. This points to p53 activation after oxidative stress being upstream of the mitochondria-mediated cell death pathway in NSCs. The p53 protein levels are kept low under normal conditions via a rapid degradation by ubiquitin-dependent mechanisms (Chowdary et al., 1994; Oren et al., 1981; Scheffner et al., 1990). The interaction of ROS with DNA causes single-, or double-strand breaks, or crosslinking, and it has been shown that these DNA damages can lead to p53 accumulation in various cell types, such as fibroblasts, Jurkat cells, HeLa cells and cardiomyocytes (Dumont et al., 1999; Gansauge et al., 1997; Renzing et al., 1996; Uberti et al., 1999; von Harsdorf et al., 1999). Depending on the cell type and injury, p53 can induce or regulate multiple cellular processes, such as cell cycle arrest, senescence and apoptosis (Clarke et al., 1993; Gudkov and Komarova, 2003; Kastan et al., 1991; Lane, 1992; Lu and Lane, 1993; Michael and Oren, 2003; Wahl and Carr, 2001; Vousden and Lu, 2002). During development p53 is highly expressed in neural cells (van Lookeren Campagne and Gill, 1998), and p53-deficient NSCs show dramatically reduced caspase-3 activation and cell death following exposure to irradiation (D'Sa-Eipper et al., 2001).

That p53 induces cell death by up-regulation of several target genes, e.g. the pro-apoptotic Bcl-2 family protein Bax, is today well known (Brady and Gil-Gomez, 1998; Jordan et al., 1997; Miyashita and Reed, 1995; Morrison et al., 2003; Xiang et al., 1998). Conversely, recent reports have suggested a novel transcription-independent role for p53 in apoptosis regulation. Upon DNA damage, p53 has been implicated in the activation of caspase-2, which in turn can induce the intrinsic mitochondria-mediated apoptotic pathway (reviewed by Zhivotovsky and Orrenius, 2005). Concurringly, we could detect activation of caspase-2 early on after DMNQ-exposure. In addition, cells pre-treated with the most selective caspase-2 inhibitor zVDVAD-fmk did exhibit significantly less apoptotic cells. Interestingly, high expression of procaspase-2 mRNA has been observed in the embryonic mouse brain, but downregulated in the adult brain (Kumar et al., 1992). Since NSCs exist in most developmental stages and even have been suggested to constitute the major cell type of the early ectoderm (Davis and Temple, 1994; Temple, 2001), caspase-2 could have a crucial role in NSC apoptosis during development. Our data suggest that oxidative stress-induced caspase-2 activation occurs upstream of the mitochondria in NSCs, and has a regulatory role in the activation of the mitochondria-mediated apoptotic pathway.

In the first two papers we reported that both aNSCs and C17.2 cells express the death receptor Fas. However, exposure to Fas-activating mAb did not induce apoptosis. We obtained similar results with the cNSCs, even when we tried to induce apoptosis with a highly effective Fas ligand construct (unpublished results). Overexpression of procaspase-8 or inhibition of FLIP did not make C17.2 cells sensitive to Fas mAb. Besides its well-characterized function as an apoptosis inducer, alternative functions for Fas have been proposed. Recently is has been shown that activation of the Fas/FasL system controls neuronal branching (Zuliani et al., 2006). Also, crosslinking of Fas has been shown to promote neurite outgrowth and acceleration of functional recovery after nerve crush injury in a caspase-8 independent manner (Desbarats et al., 2003). Activation of ERK inhibits Fas-induced apoptosis, and inhibition of ERK phosphorylation prevents Fas-induced proliferation. These data point to a direct involvement of the ERK-pathway in the signal transduction of Fas-mediated proliferation (Desbarats et al., 2003). The Fas-induced ERK phosphorylation that we reported in Paper II suggests that Fas may have a non-apoptotic function in our experimental models as well.

Even though stem cells have been used increasingly in different experimental studies, their use as a model system for developmental neurotoxicological studies is still limited. Thus, after elucidating the general apoptotic machinery in NSCs, we used them as a model to investigate the cytotoxic effects of MeHg and Mn. For more than half a century, MeHg has been known to be a public health risk. Its neurotoxic effects range from mild behavioural alterations to morbidity in both adults and infants (NRC, 2000). Developing organisms are more susceptible to MeHg as compared to adults. This high susceptibility to MeHg of the developing CNS results in serious neurological effects in the progeny, even when no signs of toxicity are observed in the mothers (Clarkson, 1972; Harada, 1978; Marsh et al., 1987). Compared to the restricted damage that occurs following MeHg-exposure in adult life, prenatal exposure results in wide-ranging adverse effects on brain development and organization, primarily related to a reduction in neural cells and altered cytoarchitecture (Choi, 1986; Choi, 1989; Choi et al., 1978; Eto et al., 1992; Geelen et al., 1990; Matsumoto et al., 1965; Mottet and Body, 1974; Takeuchi et al., 1977). Studies have shown that MeHg decreases the number of NSCs via impaired cell cycle transition and mitotic inhibition (Choi, 1989; Choi, 1991; Howard and Mottet, 1986; Matsumoto et al., 1965; Miura and Imura, 1987; Ponce et al., 1994; Rodier et al., 1984; Sager, 1988; Sager et al., 1984).

The neurodevelopmental toxicity of Mn has recently been uncovered as a significant public health concern. Epidemiologic studies of children have shown that manganese hair levels are associated with hyperactivity (Collipp et al., 1983; Pihl and Parkes, 1977). Similarly, a Canadian study (Bouchard et al., 2007) has shown that exposure to high levels of Mn in tap water results in elevated Mn hair levels in children, which are significantly associated with hyperactivity and oppositional behaviour. Additionally, as mentioned in the introduction, Wasserman and co-workers showed that Bangladeshi

children drinking well water with high concentrations of Mn have decreased intellectual functions (Wasserman et al., 2006). Moreover, studies on neonatal rats have shown a worse brain pathology and an increased brain accumulation of Mn compared to adult rats after exposure to similar doses (Chandra and Shukla, 1978; Dorman et al., 2000; Seth et al., 1977; Shukla et al., 1980).

Our results showed that NSCs, especially embryonic cNSCs, are highly sensitive to MeHg, and that cells underwent apoptotic cell death via bax oligomerization, cytochrome *c* release from the mitochondria with subsequent activation of caspase-3. In addition to caspases, a parallel calpain-dependent pathway was also activated during MeHg-induced cytotoxicity. This pathway is most likely activated by perturbation in intracellular Ca^{2+} homeostasis (Sorimachi et al., 1997), which occurs during exposure to MeHg. The formation of ROS derived from mitochondrial damage has previously been suggested in both MeHg- and Mn-induced toxicity (Kitazawa et al., 2002; LeBel et al., 1990; Sarafian and Verity, 1991), and oxidative stress also seems to play a role in our *in vitro* models, as shown by the protective effects that antioxidants had on NSCs exposed to MeHg or Mn as well as by the increase of ROS in the exposed cells. When it comes to the Mn-induced toxicity, our data showed that C17.2 NSCs were more susceptible to Mn compared to neuronal and glial cell lines, such as HT22 and D384, respectively. Interestingly, the C17.2 cell line and the primary cNSCs were almost equally susceptible to Mn in contrast to what we observed in paper III with MeHg, which was at least 10 fold more toxic in cNSCs. However, comparable to MeHg, NSCs exposed to Mn underwent apoptosis via activation of the intrinsic mitochondrial pathway. Although it was recently shown that p38 MAPK signaling is implicated in Mn-induced apoptosis in various cell types, independent or dependent of the mitochondria (El McHichi et al., 2007; Oubrahim et al., 2002; Oubrahim et al., 2001), pre-treatment with a selective p38 MAPK inhibitor did not protect the C17.2 cells from the toxic effects of Mn. The partial protection by the pan-caspase inhibitor zVAD-fmk suggests that other cellular pathways not yet identified may be involved in Mn-induced apoptosis.

In previous studies from our group investigating the apoptotic cell death machinery induced by MeHg in neuronal and astrocytoma cell lines, no caspase activation could be detected although exposed cells showed clear apoptotic morphology (Dare et al., 2001a; Dare et al., 2001b). Additionally, cell death induced by Mn and STS has been shown to be independent of mitochondrial release of cytochrome *c* and caspase-3 activation in several cell types (e.g. Belmokhtar et al., 2001; Deas et al., 1998; Oubrahim et al., 2002; Roth et al., 2000; Zhang et al., 2004). Noteworthy, MeHg and Mn, as well as STS and DMNQ, induce the activation of the intrinsic mitochondrial pathway followed by the activation of caspase-3 in our *in vitro* NSC models. Overall, our data indicate that in NSCs this pathway plays a central role in the apoptosis signaling activated by toxic stimuli.

Interestingly, levels of MeHg (2.5-5 nM), lower to than those measured in cord blood of pregnant women in the general Swedish population (0.99 μg/L) (Bjornberg et al., 2005), inhibited spontaneous neuronal differentiation. This is a remarkable finding considering that a daily exposure to 0.1 μg MeHg/kg bw, which equates to a level of MeHg in cord blood 10-fold higher than the dose used in our differentiation studies, is regarded as safe and with no risks of deleterious effects (NRC, 2000). Thus in light of our results, there seems to be a narrow margin of safety against risk of neurodevelopmental effects caused by MeHg. Hence, dietary advisories for pregnant women are necessary and justified. Moreover, considering that NSCs are also present in the adult nervous system, where they may have a role in learning, memory, and response to injuries, exposure to low levels of MeHg may have negative consequences in adulthood as well. Several factors involved in cell signaling may contribute to the observed effects of MeHg on NSCs survival and differentiation, including alterations in Notch receptor signaling, as shown by our results.

Signaling through the transmembrane receptor Notch has been implicated in a wide variety of essential cellular events, such as proliferation, migration, differentiation and neurite outgrowth (Artavanis-Tsakonas et al., 1999). Ligand-induced Notch activation results in the cleavage of the extracellular domain by ADAM/TACE/Kuzbanian metallproteases and of the intracellular domain by gamma-secretase of the receptor with subsequent translocation of the NICD to the nucleus (Ehebauer et al., 2006; Hansson et al., 2006; Hurlbut et al., 2007). In the nucleus, NICD can bind directly to the DNA-binding transcription factor CSL (CBF1, Su(H), Lag1) that attracts transcriptional activators and co-activators of genes responsible for e.g. repressive effects neuronal differentiation (Ehebauer et al., 2006; Hurlbut et al., 2007; Kageyama et al., 2005). Mercurial compounds, such as p-aminophenylmercuric acetate, have previously been shown to regulate ADAM metalloprotease activity (Bland et al., 2003), and interestingly, in a recent study micromolar concentrations of MeHg were reported to induce Notch signaling and activate downstream transcriptional targets in *Drosophila melanogaster* neuronal-like cell lines (Bland and Rand, 2006). As shown by the study reported in paper III, very low doses of MeHg (1-10 nM) efficiently inhibit neuronal differentiation of cNSCs. In paper VI we investigated whether Notch signaling was activated in cNSCs by MeHg, and if such putative activation could be involved in the inhibition of neuronal differentiation. By using a fluorescent-based reporter construct, we found that Notch signaling was activated in mammal NSCs by low subcytotoxic nanomolar concentrations of MeHg. These data were further strengthened by immunoblotting of NICD, which showed a dose-dependent increase after MeHg exposure. Our data support the finding of Bland and Rand that found activation of the Notch signaling pathway in *Drosophila* CNS cells exposed to micromolar concentration of MeHg (Bland and Rand, 2006). Additionally, we found that the potent metalloprotease inhibitor GM6001 prevented the inhibition of neuronal formation induced by MeHg. All together our data point to MeHg activating ADAM

metalloproteases that induce the Notch signaling pathway with subsequent repression of neuronal differentiation of NSCs.

CONCLUSION

In conclusion, our data shows that STS and the oxidative stress-inducer DMNQ cause apoptosis via the mitochondrial pathway in NSCs. The release of cytochrome *c* from the mitochondria is associated with activation of caspase-3, nuclear condensation, chromatin fragmentation, and other typical apoptotic features. The DMNQ-generated formation of ROS causes p53 accumulation with subsequent activation of caspase-2. The latter plays a regulatory role in the induction of the mitochondrial apoptotic pathway. Although the Fas receptor is expressed, the death receptor pathway is not operative in NSCs. Fas-induced ERK phosphorylation suggests that in NSCs Fas may function as a mediator of growth rather than death. NSCs are highly sensitive to the toxic effects of MeHg and Mn, and cells undergo apoptosis via activation of the mitochondria-mediated caspase-dependent apoptotic pathway activated most likely by oxidative stress. In parallel, MeHg induces an additional cell death pathway involving the activation of calpains. Also Mn seems to exert its toxicity via additional pathways, which need to be clarified. Overall, when we used our NSC models to study *de facto* environmental contaminants, which exert their toxicity via oxidative stress, a similar activation of the intrinsic mitochondrial cell death pathway could be observed.

Our results indicate that MeHg can exert its detrimental effects on the developing nervous system via activation of Notch signaling and the subsequent inhibition of neuronal differentiation. Future studies aimed at establishing whether the adverse developmental effects seen *in vivo* after MeHg exposure can be related to changes in Notch signaling will move forward the comprehension of the impact of MeHg on neurodevelopmental disorders. The adverse effects of low concentrations of MeHg, similar to human fetal exposure, on spontaneous neuronal differentiation of NSCs point to the need of further investigations of NSCs exposed to doses lower than the estimated reference dose of neurotoxic substances. By confirming the *in vivo* data showing high sensitivity of the developing nervous system to MeHg, our study shows that cultures of NSCs are good *in vitro* models for developmental neurotoxicity studies to identify the potential neurodevelopmental effects of toxic substances, and the effects that extrinsic factors play in the cell biology of NSCs.

Although epidemiological studies have a critical role in providing evidence for the association between toxicants and neurodevelopmental disorders, *in vitro* experimental studies aimed at dissecting the cellular and molecular mechanisms of toxicity are needed. The difference in sensitivity to MeHg and Mn that we have observed in NSCs as compared to other neural cells, further strengthens the fact that multiple *in vitro* cellular models, including NSCs, are required for an accurate assessment of the toxic effects of suspected neurotoxic substances.

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